

SEARCH FOR IRON REDOX ABILITIES IN EXTREMELY ACIDOPHILIC MICROORGANISMS AND THEIR USE IN MINERAL PROCESSING

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<https://hdl.handle.net/2324/1807014>

出版情報 : 九州大学, 2016, 博士 (工学), 課程博士
バージョン :
権利関係 :





**Search for iron redox abilities in extremely acidophilic
microorganisms and their use in mineral processing**

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A thesis submitted to
Kyushu University
for the degree of
Doctor of Engineering

March 2017

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Abstract

The thesis consists of investigations of iron redox abilities of extremely acidophilic microorganisms as fundamental studies (chapter 3 and 4) and their utilization to mineral processing as application studies (chapter 5 to 8). The latter includes reductive bioleaching of Indonesian Ni-limonite ore and chalcopyrite concentrate, and oxidative bioleaching of chalcopyrite concentrate in silver catalyzed or redox potential-controlled systems.

In chapter 1, background and objectives of this work are provided especially focusing on importance of microbiological iron redox transformation and its utilization to mineral processing. Previous studies related to the present work were reviewed and discussed in this chapter.

In chapter 2, methodologies used in this work are described.

In chapter 3, for the purpose of utilizing microbial Fe(III) reduction for reductive bioleaching of Fe(III)-containing minerals and for bioremediation of heavy metal polluted-environments, useful Fe(III)-reducing acidophiles were searched from Chinoike Jigoku (“Blood Pond Hell”) hot spring located in Beppu, on Kyushu Island of Japan. This is because the hot spring features acidic pHs and a large amount of ferric mineral in sedimentary layers. The presence of Fe metabolizing acidophiles was implied based on the 16S rRNA gene analysis. Successive enrichment cultivation with Fe(III) under micro-aerobic conditions resulted in isolation of an acidophilic archaeon, *Sulfolobus* (S.) sp. GA1 (with 99.7% 16S rRNA gene sequence identity with *S. shibatae* B12^T). Strain GA1 showed unique characteristics (unlike other *Sulfolobus* spp.), capable of reducing Fe(III), and incapable of oxidizing S⁰. The strain was found to reduce extremely toxic Cr(VI) to less toxic/soluble Cr(III).

The facts revealed in chapter 3 motivated us to further investigation of general trend of Fe redox transformation abilities in six representative *Sulfolobus* spp. **in chapter 4**. The degree of Fe(II)-oxidizing abilities were found to differ largely between the strains tested. Four strains (*S. tokodaii* 7, *S. solfataricus* P1, *S. shibatae* B12 and *Sulfolobus* sp. GA1) were capable of anaerobic growth via Fe(III) respiration. All strains other than *Sulfolobus* sp. GA1 displayed Fe(III)-reducing abilities in growth-uncoupled cell suspensions: Nonetheless, Fe(III)-reducing ability in each strain

responded significantly differently to the oxygen level. The trend of Fe(III) reduction in six strains did not correspond to that of Cr(VI), implying different mechanisms being involved in Fe(III) and Cr(VI) reduction in *Sulfolobus* spp. tested. The information on Fe redox transformation abilities by *Sulfolobus* spp. contributed to better understanding of Fe redox cycles in natural environments, and provided its potential applicability to mineral processing such as biohydrometallurgical approaches.

In chapter 5, screening of useful Fe(III)-reducing acidophiles was carried out based on their effectiveness in soluble Fe(III) reduction and subsequent dissolution of chemically synthesized goethite. The selected Fe(III)-reducing acidophiles were then tested for reductive bioleaching of Indonesian Ni-limonite ore (mainly consisting of goethite). Dissolution behaviors of Ni and Fe from the limonite ore were linearly correlated and Ni extraction was found to be sensitive to solution redox potentials, total soluble Fe concentrations, and solution pHs. Ni extraction continued by refreshing bioleaching media to remove dissolved metals: The maximum Ni extraction (within 30 days) of 55 or 41% were marked at the end of the 3rd step using cell suspensions of *S. tokodaii* 7 or *Sb. acidophilus* YTF1, respectively.

In chapter 6, applicability of the reductive bioleaching approach to refractory primary copper sulfides, chalcopyrite (CuFeS_2), was investigated. However, no noticeable improvement in Cu recovery was observed. This was likely caused by higher refractoriness of chalcopyrite than Fe(III) oxides (such as goethite), hindering its acid dissolution.

In chapter 7, catalytic effect of silver on chalcopyrite bioleaching was investigated. When tested with silver sulfate, microbial oxidizing capabilities of Fe(II) and S^0 were both inhibited by the presence of silver catalyst. Unexpectedly, after silver sulfate was added at day 10, sterile controls showed higher Cu recovery (52% at day 42) than that in mixed cultures (43% at day 42). This was likely caused by passivation layer of S^0 accumulation that formed at higher solution redox potentials before the addition of silver sulfate to mixed cultures.

In chapter 8, potential utility of the microbiological solution redox potential control during chalcopyrite bioleaching was investigated using mixed cultures of moderately thermophilic acidophiles. Utilization of different mixed cultures showing different degrees of Fe(II)-oxidizing capability resulted in different levels of E_{normal} and

Cu leaching rate. Final Cu recovery in mixed cultures of *Acidithiobacillus (At.) caldus* KU plus *Sb. acidophilus* YTF1 marked 75% by day 70, while that in mixed cultures of *At. caldus* KU plus *Acidiplasma* sp. Fv-Ap was 49%. The above difference in Cu recovery was attributed to the E_{normal} level: $0 < E_{\text{normal}} < 1$ was maintained throughout the bioleaching test in the former, whereas E_{normal} rapidly exceeded 1 in the latter. Effectiveness of chalcopyrite dissolution during the test was discussed based on the kinetic study. Chalcopyrite bioleaching was found to be controlled by “surface reaction” at $0 < E_{\text{normal}} < 1$, while that was controlled by “diffusion through product layers” (e.g., jarosite) at $E_{\text{normal}} > 1$. Consequently, different kinetics involved in chalcopyrite bioleaching were found to lead to either facilitation ($0 < E_{\text{normal}} < 1$; using “weak” Fe(II)-oxidizing microbes) or hindrance ($E_{\text{normal}} > 1$; using “strong” Fe(II)-oxidizing microbes) of the mineral dissolution. The Cu leaching rate was shown to be maximized at the E_{normal} value of approx. 0.3.

In chapter 9, conclusions and recommendations for future work are provided.

The flow from fundamental to application studies in this work provided us with new aspects and approaches in resource engineering, leading to further development of biological mineral processing technologies.

要旨

本博士論文は、超好酸性微生物における鉄酸化/還元能の探索及び比較（第3、4章；基礎研究）、さらにそれらを利用した鉱物処理（第5-8章；応用研究）に関する研究論文である。後者の応用研究では、インドネシア産天然リモナイト鉱/黄銅鉱精鉱における還元的バイオリーチング、及び黄銅鉱精鉱の酸化的バイオリーチングにおける銀添加/微生物学的溶液電位制御の影響について研究を行った。

第1章では、微生物学的鉄酸化/還元能及びそれらの鉱物処理への応用に関する研究背景について記述した。また、それらの先行研究についてのまとめも行った。

第2章では、本博士論文中の実験における一般的手順及び分析方法等についてまとめた。

第3章では、三価鉄含有鉱物の還元的バイオリーチングや重金属バイオレメディエーション等への適応可能が期待される、好酸性鉄還元微生物の探索及び単離を目的とした。対象探索地としては、多量の鉄含有堆積物が確認され、さらにその鉱水が酸性であるという特徴を持つ、血の池地獄温泉（大分県別府市）を選定した。16SrRNA 遺伝子配列の一部を基にした、原位置における微生物群衆構造解析では、複数種の鉄還元/酸化細菌の存在が確認された。集積培養を行うことによって、最終的に鉄還元古細菌 *Sulfolobus* sp. GA1 株（*S. shibatae* B12 株の 16SrRNA 遺伝子と 99.7%の相同性を示す）が単離された。GA1 株は、鉄還元能を示したが硫黄酸化能を示さず、他の *Sulfolobus* 属の特徴と異なった特色を示した。また、GA1 株は毒性重金属である六価クロムの還元能も有することが分かった。

第4章では、第3章での単離株 GA1 株を含めた *Sulfolobus* 属 6 株（*S. shibatae* B12 株、*S. tokodaii* 7 株、*S. metallicus* Kra23 株、*S. acidocaldarius* 98-3 株、*S. solfataricus* P1 株、及び *Sulfolobus* sp. GA1 株）において、その鉄酸化/還元能の比較を定量的に行った。それらの鉄酸化能は、それぞれの株によって大きく異なり、*S. metallicus* Kra23 株が最も優れた鉄酸化速度を示した。絶対嫌気条件下において異化的鉄還元による生育が見られたのは、*S. shibatae* B12 株、*S. tokodaii* 7 株、*S. solfataricus* P1 株、及び *Sulfolobus* sp. GA1 株であった。6 種の *Sulfolobus* 株の鉄還元速度（効率）は、溶存酸素濃度に特に影響を受け、各株によってその傾向は異なった。各株における六価クロム還元挙動はそれらの鉄還元挙動とは完全に関連していなかった。本研究にて新たに確認された、*Sulfolobus* 属における鉄還元能及び六価クロム還元能は、鉄含有鉱物の還元的バイオリーチングや重金属バイオレメディエーション等への適応可能性が期待される。

第 5 章では、好酸性鉄還元微生物のスクリーニングで有能な鉄還元能を示した微生物株 *S. tokodaii* 7 株（至適温度 70 度）及び *Sulfobacillus* sp. YTF1 株（至適温度 45 度）を用いた還元的バイオリーチングによる、主に goethite（三価鉄含有鉱物）から成る天然リモナイト鉱のニッケル浸出率の向上を目的とした。還元的バイオリーチングによる天然リモナイト鉱からの鉄の溶出に伴うニッケルの浸出が確認され、それらの浸出率の高い相関性を示した。ニッケル浸出効率は溶液電位及び pH に強く依存することが分かった。また、溶存した過剰な鉄を取り除くため、バイオリーチング後の浸出液を新たなものと入れ替えることで、リモナイト鉱からのさらなるニッケル浸出が確認され、3 ステップ後（全 30 日間）には *S. tokodaii* 7 株（70 度）及び *Sb. acidophilus* YTF1 株（45 度）の懸濁液の系において、それぞれ 55 及び 41% のニッケル回収率を達成した（無菌系では約 30% 程度）。

第 6 章では、黄銅鉱（ CuFeS_2 ）精鉱に対する還元的バイオリーチングの適応可能性を評価したが、その難処理性から銅浸出率の向上は確認されなかった。

第 7 章では、黄銅鉱精鉱のバイオリーチングにおける、硫化銀/硫酸銀の添加による銅浸出効率の向上を目的として実験を行った。実験開始後 10 日での銀添加により、その後の微生物学的鉄/硫黄酸化挙動に一部阻害が見られた。さらに、42 日経過後の有菌系における銅浸出率（43%）を無菌系におけるそれ（52%）が上回った。この原因としては、有系においては、銀添加した際の溶液電位が約 800 mV vs. SHE と高かったことにより、黄銅鉱表面に jarosite や元素硫黄の不動態層が生成したことが挙げられる。

第 8 章では、黄銅鉱バイオリーチングにおける微生物学的溶液電位制御の銅浸出率への影響について、特に、“黄銅鉱浸出の溶液電位依存性”及び“熱力学的反応”の観点から考察を行った。本実験では、異なる鉄酸化能を有する中度高熱好酸性鉄酸化微生物を用いることによって、黄銅鉱バイオリーチング中の溶液電位の制御を試みた。また、溶液電位は E_{normal} を用いて評価した。*Acidithiobacillus* (*At.*) *calculus* KU 株と *Sb. acidophilus* YTF1 株を用いたバイオリーチングの系では、最終銅浸出率それぞれ 75%（70 日後）を達成した。一方で、*At. calculus* KU 株と *Acidiplasma* sp. Fv-Ap 株を用いたバイオリーチングの系では、それは 49%（70 日後）であった。この銅浸出率の差は、以下の 2 点によって説明できる。

（1）前者の系では実験を通して $0 < E_{\text{normal}} < 1$ を満たしており、黄銅鉱の浸出が中間生成物輝銅鉱（ Cu_2S ）を介して起こった。（2）一方、後者の系では実験を通して $1 < E_{\text{normal}}$ を満たしており、黄銅鉱は三価鉄による酸化溶解によって浸出した。さらに、それらの浸出機構はそれぞれ、表面反応速度律速または不動態層による拡散律速であることが分かり、前者においてより高い反応速度定数が示された。本章では、黄銅鉱の溶解が溶液電位

に強く依存することが分かり、本バイオリーチングの実験条件における黄銅鉱浸出効率を基に判断した E_{normal} の最適値は、およそ 0.3 であった。

第 9 章では、結言及び総括、また、今後の課題等について記述した。

本博士論文では、超好酸性微生物における鉄酸化/還元能の探索及び比較に関して第 3 及び 4 章を基礎研究、さらにそれらを利用した鉱物処理に関して第 5 から 8 章を応用研究として行った。これらの一連の研究は、資源工学、特に微生物学的湿式精錬におけるさらなる発展に寄与するものであると考える。

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List of Abbreviations

<i>A.</i>	<i>Acidiphilium</i>
<i>Ac.</i>	<i>Acidocella</i>
<i>Ali.</i>	<i>Alicyclobacillus</i>
<i>Am.</i>	<i>Acidimicrobium</i>
<i>Ap.</i>	<i>Acidiplasma</i>
<i>At.</i>	<i>Acidithiobacillus</i>
EPMA	Electron Probe Micro Analyzer
<i>Fm.</i>	<i>Ferrimicrobium</i>
<i>Fp.</i>	<i>Ferroplasma</i>
HBS	Heterotrophic basal salts
ICP-AES	Inductively Coupled Plasma-Atomic Emission Spectroscopy
<i>L.</i>	<i>Leptospirillum</i>
<i>M.</i>	<i>Metallosphaera</i>
PCR	Polymerase Chain Reaction
SEM	Scanning Electron Microscope
<i>S.</i>	<i>Sulfolobus</i>
<i>Sb.</i>	<i>Sulfobacillus</i>
TOC	Total Organic Carbon Analyzer
XAFS	X-ray Absorption Fine Structure
XANES	X-ray Absorption Near-Edge Structure
XPS	X-ray Photoelectron Spectroscopy
XRD	X-ray Diffraction
XRF	X-ray Fluorescence

Chapter 1

Introduction

1.1 Search for iron redox abilities in extremely acidophilic microorganisms

Iron (Fe) is the most abundant element on the earth. Iron ions in soluble phase are more bio-available at lower pHs where extremely acidophilic microorganisms are active, given that iron ions in both ionic forms (ferrous and ferric) are more soluble at lower pHs rather than at neutral pHs, and that extremely acidic environments are often generated by oxidation of sulfide minerals (e.g., pyrite; the most abundant sulfides in the lithosphere) caused by both natural phenomenon and human activity such as mining activity (Johnson et al 2012). Therefore, iron redox transformation via microbiological Fe oxidizing/ reducing activity plays an important role in extremely acidic environments. Study on search for iron redox abilities in extremely acidophilic microorganisms therefore contributes to better understandings of geochemical Fe cycles in highly acidic environments.

Iron is also the most abundant transition metal in the lithosphere. Therefore, its redox transformation has been recognized to be available to many techniques for environmental remediation of polluted aqueous systems (e.g., treatment of acid mine drainage, remediation of toxic metals) and mineral processing in hydrometallurgical processes (e.g., reductive dissolution of oxide minerals, chemical and biological leaching of sulfide minerals). Since solution pHs in these polluted aqueous systems and hydrometallurgical processes are often highly acidic, further investigations of iron redox abilities in extreme acidophiles is meaningful for further development of these techniques.

1.2 Utilization of microbial Fe(III) reduction to mineral processing techniques

1.2.1 Nickel recovery from laterite ores via reductive bioleaching using Fe(III)-reducing acidophiles

Since around 2010, a microbiological approach, reductive bioleaching using Fe(III)-reducing acidophiles, has newly been proposed for Ni extraction from laterite ore (especially from limonitic ores; Hallberg et al 2011, Johnson 2012). The new approach can be an alternative technique to conventional commercial processes of Ni-laterite (e.g., pyrometallurgical roasting process, followed by hydrometallurgical ammoniacal chemical leaching (Caron 1950) and high pressure acid leaching (HPAL; Carlson et al 1961)), and even to possibly applicable biological/chemical Ni extraction techniques using inorganic or organic acid (Tzeferis et al 1994, Valix et al 2001a, 2001b, Le et al 2006, Coto et al 2008, Behera et al 2011, Astuti et al 2015, 2016a, 2016b). Mechanism of reductive bioleaching is discussed in 1.2.3, after previous studies on Ni extraction via hydrometallurgical techniques are reviewed in 1.2.2.

1.2.2 Previous studies on Ni recovery from laterite ores via hydrometallurgical techniques

Nickel production and demand have continuously increased for the last six decades (Dalvi et al 2004). Given that depletion of high grade nickel sulfide ores has been recognized as the problem, and that approximately 70% of world Ni resources are presented in laterites, one of the oxide ores, which are widely distributed in tropical area, technical development of Ni recovery from laterite ores has attracting increasing interest to achieve stable supply of the metal resource. Laterite deposits generally consist of layers of the limonite, the saprolite, and the h from the top to the bottom layers. Limonite is a homogeneous ore as the top layer which is mainly composed of ferric oxides, typically as goethite ($\text{FeO}(\text{OH})$) often associating with Ni (Golightly 1981, Landers et al 2009). Saprolite is distinguished by a rich magnesium and silicate oxide matrix from limonite (Valix et al 2001b). As is described above, hydrometallurgical Ni extraction techniques with lower operating cost and less waste amount is regarded as a possible alternative approach to conventional commercialized pyrometallurgical and hydrometallurgical processes

Table 1.1 Summary of Ni extraction techniques via organic/inorganic acid leaching from laterite ores.

Acid types	Laterite types	Initial pH	Temp. (°C)	Ni leached (%)	Periods (day)	Pulp density (%)	Others	Ref.
0.6 M citrate	Saprolite	2	30	5	32	10		Valix et al 2001
0.6 M citrate	Limonite	2	30	20	32	10		Valix et al 2001
0.5 M sulfate	Limonite (Litharakia, Euboea island, Greece)	0.34	30	76	50	10		Tzeferis et al 1994
0.5 M citrate	Limonite (Litharakia, Euboea island, Greece)	1.99	30	52	50	10		Tzeferis et al 1994
0.1 M citrate	Limonite (Litharakia, Euboea island, Greece)	2.55	30	23	50	10		Tzeferis et al 1994
0.2 M citrate	Limonite (Litharakia, Euboea island, Greece)	2.29	30	29	50	10		Tzeferis et al 1994
0.3 M citrate	Limonite (Litharakia, Euboea island, Greece)	2.14	30	38	50	10		Tzeferis et al 1994
0.4 M citrate	Limonite (Litharakia, Euboea island, Greece)	2.06	30	40	50	10		Tzeferis et al 1994
0.5 M acetate	Limonite (Litharakia, Euboea island, Greece)	3.88	30	4.8	50	10		Tzeferis et al 1994
0.25 M citrate, 0.25 M sulfate	Limonite (Litharakia, Euboea island, Greece)	1.12	30	58	50	10		Tzeferis et al 1994
0.25 M acetate, 0.25 M sulfate	Limonite (Litharakia, Euboea island, Greece)	0.79	30	33	20	10		Tzeferis et al 1994
0.5 M lactate	Limonite (Litharakia, Euboea island, Greece)	2.55	30	5.5	20	10		Tzeferis et al 1994

continued

Acid types	Laterite types	Initial pH	Temp. (°C)	Ni leached (%)	Periods (day)	Pulp density (%)	Others	Ref.
0.25 M lactate, 0.25 M sulfate	Limonite (Litharakia, Euboea island, Greece)	0.74	30	41	20	10		Tzeferis et al 1994
0.5 M oxalate	Limonite (Litharakia, Euboea island, Greece)	1.1	30	2.8	20	10		Tzeferis et al 1994
0.25 M oxalate, 0.25 M sulfate	Limonite (Litharakia, Euboea island, Greece)	0.41	30	24	20	10		Tzeferis et al 1994
0.25 M oxalate, 0.25 M citrate	Limonite (Litharakia, Euboea island, Greece)	1.02	30	2.8	20	10		Tzeferis et al 1994
0.4 M oxalate, 0.1 M citrate	Limonite (Litharakia, Euboea island, Greece)	0.66	30	2.8	20	10		Tzeferis et al 1994
1 M citrate	Limonite (Sulawesi, Indonesia)	1.8	RT	27	14	5		Mubarok et al 2011
1 M oxalate	Limonite (Sulawesi, Indonesia)	0.8	RT	22	14	5		Mubarok et al 2011
1 M lactate	Limonite (Sulawesi, Indonesia)	2	RT	32	14	5		Mubarok et al 2011
1 M acetate	Limonite (Sulawesi, Indonesia)	2.3	RT	9.7	7	5		Mubarok et al 2011
1 M citrate	Saprolite (Sulawesi, Indonesia)	1.8	RT	94	21	5		Mubarok et al 2011
1 M oxalate	Saprolite (Sulawesi, Indonesia)	0.8	RT	7.9	7	5		Mubarok et al 2011
1 M lactate	Saprolite (Sulawesi, Indonesia)	2	RT	45	14	5		Mubarok et al 2011

continued

Acid types	Laterite types	Initial pH	Temp. (°C)	Ni leached (%)	Periods (day)	Pulp density (%)	Others	Ref.
1 M acetate	Saprolite (Sulawesi, Indonesia)	2.3	RT	31	14	5		Mubarok et al 2011
1 M citrate	Saprolite (Halmahera, Indonesia)	-	30	43	15	20		Astuti et al 2016
1 M sulfate	Saprolite (Halmahera, Indonesia)	0.62	30	72	15	20		Astuti et al 2016
1 M nitrate	Saprolite (Halmahera, Indonesia)	0.52	30	39	15	20		Astuti et al 2016
1 M hydrochloride	Saprolite (Halmahera, Indonesia)	0.5	30	40	15	20		Astuti et al 2016
1 M oxalate	Saprolite (Halmahera, Indonesia)	-	30	3	15	20		Astuti et al 2016
1 M lactate	Saprolite (Halmahera, Indonesia)	-	30	35	15	20		Astuti et al 2016
0.25 M sulfate, 0.75 M citrate	Saprolite (Halmahera, Indonesia)	-	30	60	15	20		Astuti et al 2016
0.5 M sulfate, 0.5 M citrate	Saprolite (Halmahera, Indonesia)	-	30	71	15	20		Astuti et al 2016
0.75 M sulfate, 0.25 M citrate	Saprolite (Halmahera, Indonesia)	-	30	81	15	20		Astuti et al 2016
1 M citrate	Saprolite (Sulawesi, Indonesia)	-	30	72	15	20		Astuti et al 2016
1 M sulfate	Saprolite (Sulawesi, Indonesia)	0.6	30	72	15	20		Astuti et al 2016

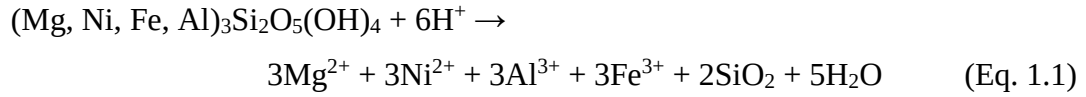
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Acid types	Laterite types	Initial pH	Temp. (°C)	Ni leached (%)	Periods (day)	Pulp density (%)	Others	Ref.
1 M nitrate	Saprolite (Sulawesi, Indonesia)	0.53	30	45	15	20		Astuti et al 2016
1 M hydrochloride	Saprolite (Sulawesi, Indonesia)	0.49	30	43	15	20		Astuti et al 2016
1 M oxalate	Saprolite (Sulawesi, Indonesia)	-	30	ND	15	20		Astuti et al 2016
1 M lactate	Saprolite (Sulawesi, Indonesia)	-	30	38	15	20		Astuti et al 2016
0.25 M sulfate, 0.75 M citrate	Saprolite (Sulawesi, Indonesia)	-	30	65	15	20		Astuti et al 2016
0.5 M sulfate, 0.5 M citrate	Saprolite (Sulawesi, Indonesia)	-	30	73	15	20		Astuti et al 2016
0.75 M sulfate, 0.25 M citrate	Saprolite (Sulawesi, Indonesia)	-	30	65	15	20		Astuti et al 2016
1 M citrate	Saprolite (Halmahera, Indonesia)	-	30	49	15	20	Particle size < 75 µm	Astuti et al 2016
1 M citrate	Saprolite (Halmahera, Indonesia)	-	40	69	15	20	Particle size < 75 µm	Astuti et al 2016
1 M citrate	Saprolite (Halmahera, Indonesia)	-	60	69	15	20	Particle size < 75 µm	Astuti et al 2016
0.5 M citrate	Saprolite (Halmahera, Indonesia)	-	40	67	15	20	Particle size < 75 µm	Astuti et al 2016
0.1 M citrate	Saprolite (Halmahera, Indonesia)	-	40	25	15	20	Particle size < 75 µm	Astuti et al 2016

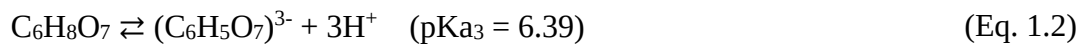
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Acid types	Laterite types	Initial pH	Temp. (°C)	Ni leached (%)	Periods (day)	Pulp density (%)	Others	Ref.
1 M citrate	Saprolite (Halmahera, Indonesia)	-	40	69	15	20	Particle size; 75-150 µm	Astuti et al 2016
1 M citrate	Saprolite (Halmahera, Indonesia)	-	40	72	15	20	Particle size; 212-355 µm	Astuti et al 2016
1 M citrate	Saprolite (Halmahera, Indonesia)	-	40	54	15	20	Particle size; 350-855 µm	Astuti et al 2016
1 M citrate	Saprolite (Sulawesi, Indonesia)	-	30	65	15	20	Particle size < 75 µm	Astuti et al 2016
1 M citrate	Saprolite (Sulawesi, Indonesia)	-	40	80	15	20	Particle size < 75 µm	Astuti et al 2016
1 M citrate	Saprolite (Sulawesi, Indonesia)	-	60	80	15	20	Particle size < 75 µm	Astuti et al 2016
0.5 M citrate	Saprolite (Sulawesi, Indonesia)	-	40	55	15	20	Particle size < 75 µm	Astuti et al 2016
0.1 M citrate	Saprolite (Sulawesi, Indonesia)	-	40	20	15	20	Particle size < 75 µm	Astuti et al 2016
1 M citrate	Saprolite (Sulawesi, Indonesia)	-	40	92	15	20	Particle size; 75-150 µm	Astuti et al 2016
1 M citrate	Saprolite (Sulawesi, Indonesia)	-	40	95	15	20	Particle size; 212-355 µm	Astuti et al 2016
1 M citrate	Saprolite (Sulawesi, Indonesia)	-	40	76	15	20	Particle size; 350-855 µm	Astuti et al 2016

Improvement in Ni extraction techniques from laterite ores using several inorganic/organic acids (e.g., citrate, acetate, lactate, oxalate, sulfate, nitrate, hydrochloride, etc.) was studied by many research groups and summarized in Table 1.1. According to reports comparing Ni extraction efficiency via different organic acids, citrate has been recognized as an acid which generally gives higher Ni recovery from saprolite mainly consisting of serpentine, magnesium rich silicate mineral. Many research groups concluded a leaching mechanism of Ni ions from saprolite ore, especially from serpentine phase, using inorganic/organic acids as follows (Astuti et al 2016b);



In this leaching mechanism, acidity is important. Protons (H^+) is provided by dissociation of inorganic/organic acids. Displacement of metal cations in the mineral matrix by protons occurs, resulting acid leaching of metal ions into solution phase. When using organic acids, but not inorganic, another mechanism that facilitates leaching of metal ions, occurs: Organic acids can form soluble metal-ligand complexes by chelation (McKenzie et al 1987). In the case of citrate, the chelation reaction occurs as follows (Behera et al 2011);



Owing to the chelation reaction, un-complexed Ni ions are consumed, inducing shift of its chemical equilibrium, as follows;



Tzeferis et al (1994) concluded that mixture of citrate and sulfate as leaching acid extract Ni more effectively than either citrate or sulfate alone at the same initial free

proton concentration, indicating the chelating reaction with citrate is additive Ni leaching mechanism to acid leaching of serpentine.

Microbially-produced organic/inorganic acids are considered as alternative materials to externally-provided acids. Metal extraction techniques from oxides and silicate via bioleaching are divided into two types; bioleaching via heterotrophic or autotrophic microorganisms. In the former, *Aspergillus* spp. and *Penicillium* spp. are recognized as the most important organic acid-producers, while *At. thiooxidans* is used as sulfate-producer in the latter (Franz et al 1991, Valix et al 2001a, 2001b, Le et al 2006, Coto et al 2008). Ni extraction efficiency via bioleaching and its leaching conditions are summarized in Table 1.2. While relatively higher Ni extraction from laterite tailing after CARON process was reported, its efficiency from un-processed saprolite and limonite is still relatively low; up to around 30% within 20 to 60 days, which might have been caused by lower concentration of microbially-produced organic/inorganic acids.

Table 1.2 Summary of Ni extraction techniques via bioleaching of laterite ores.

Microorganisms	Laterite types	Initial pH	Temp. (°C)	Ni leached (%)	Periods (day)	Pulp density (%)	Others	Ref.
<i>Penicillium funiculosum</i>	Saprolite	4.5	30	14	59	10		Valix et al 2001
<i>Penicillium funiculosum</i>	Limonite	4.5	30	8.8	59	10		Valix et al 2001
<i>Aspergillus niger</i>	Saprolite	4.7	30	3.2	59	10		Valix et al 2001
<i>Aspergillus niger</i>	Limonite	4.5	30	5.7	44	10		Valix et al 2001
<i>Penicillium funiculosum</i>	Saprolite	-	30	14	59	10		Valix et al 2001
<i>Penicillium funiculosum</i>	Limonite	-	30	8	59	10		Valix et al 2001
<i>Aspergillus niger</i>	Saprolite	-	30	34	59	10		Valix et al 2001
<i>Aspergillus niger</i>	Limonite	-	30	6	43	10		Valix et al 2001
<i>Aspergillus niger</i> O5	Laterite tailings (CARON process)	5	30	7	9	5		Coto et al 2008
<i>At. thiooxidans</i> DSM11478	Laterite tailings (CARON process)	1.5	30	93	15	2.5		Coto et al 2008
<i>At. thiooxidans</i> DSM11478	Laterite tailings (CARON process)	1.5	30	90	15	5		Coto et al 2008
<i>At. thiooxidans</i> DSM11478	Laterite tailings (CARON process)	1.5	30	71	15	10		Coto et al 2008

continued

Microorganisms	Laterite types	Initial pH	Temp. (°C)	Ni leached (%)	Periods (day)	Pulp density (%)	Others	Ref.
Inorganic acid produced by <i>At.thiooxidans</i>	Laterite tailings (CARON process)	0.9	60	61	6	10	Cells removed	Coto et al 2008
<i>Aspergillus foetidus</i>	Saprolite (New Caledonia)	-	30	19	13	1		Le et al 2006
<i>Aspergillus foetidus</i>	Saprolite (New Caledonia)	-	30	28	18	1	Multi metal-tolerant (438 ppm)	Le et al 2006
<i>Aspergillus foetidus</i>	Saprolite (New Caledonia)	-	30	29	13	1	Multi metal-tolerant (1095 ppm)	Le et al 2006

1.2.3 Reviews of reductive bioleaching of ferric oxides using Fe(III)-reducing acidophiles

As was described previously, Ni laterites are divided into two types; limonite (consisting mainly of goethite (FeOOH)) and saprolite (known as serpentine ((Ni, Mg, Fe)₃Si₂O₅(OH)₄) rich laterite). Since acid leaching using citrate through Ni-citrate chelation provides greater Ni extraction efficiency for serpentine rather than for goethite, further development of Ni extraction techniques from limonite ores is necessary to maximize Ni recovery from laterite ores including the both types.

Recently, reductive bioleaching of ferric oxides (e.g., goethite) using Fe(III)-reducing acidophiles has been proposed, as was mentioned previously, (Hallberg et al 2011, Johnson 2012, Johnson et al 2013). They have suggested a dissolution mechanism of goethite mineral via reductive bioleaching as follows; (i) acid dissolution of goethite via chemical equilibrium of Fe(III) in between solid and soluble phases (Eq. 1.5), and (ii) reduction of soluble Fe(III) to Fe(II) by Fe(III)-reducing acidophiles (Eq. 1.6), promoting dissolution of goethite, followed by extraction of Ni ions. Scheme of its dissolution mechanism is shown in Fig. 1.1.

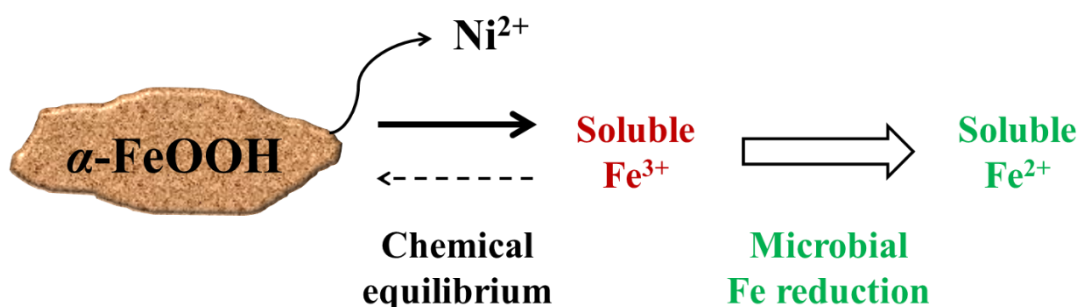


Fig. 1.1 Scheme of reductive bioleaching of goethite for Ni extraction.

Ni recovery from limonite ore via reductive bioleaching using microbial Fe(III) reduction has been investigated (Plessia et al 2011, Marrero et al 2015), and summarized in Table 1.3. Further studies on the technique surely contributes to stable supply of Ni to industrialized society.

Table 1.3 Summary of Ni extraction from limonite ores via reductive bioleaching at acidic pHs and 30°C.

Microorganisms	Laterite types	Ni leached (%)	Fe leached (%)	Periods (day)	Pulp density (%)	Conditions	Others	Ref.
<i>At. ferrooxidans</i>	Milled (< 1 mm) limonite (Western Australia)	82	63	30	5.625	Anoxic	pH maintained	Hallberg et al 2011
<i>At. ferrooxidans</i>	Crushed (< 6 mm) limonite (Western Australia)	82	58	20	5.625	Anoxic	pH maintained	Hallberg et al 2011
<i>At. ferrooxidans</i>	Milled (< 1 mm) limonite (Western Australia)	82	73	26	5.625	Anoxic	5 mM citrate, pH maintained	Hallberg et al 2011
<i>At. ferrooxidans</i>	Laterite tailings (CARON process)	53	-	11	10 (2.5% pulp added each time at 0, 8, 24, and 48 h)	Aerobic		Marrero et al 2015
A mixed culture of <i>At. ferrooxidans</i> and <i>At. thiooxidans</i>	Laterite tailings (CARON process)	56	-	11		Aerobic		Marrero et al 2015
A mixed culture of <i>At. ferrooxidans</i> and <i>At. thiooxidans</i>	Laterite tailings (CARON process)	53	-	11		Aerobic (3 days) → anaerobic		Marrero et al 2015
A mixed culture of <i>At. ferrooxidans</i> and <i>At. thiooxidans</i>	Laterite tailings (CARON process)	58	-	11		Anaerobic		Marrero et al 2015

1.3 Utilization of microbial Fe(II) oxidation to mineral processing techniques

1.3.1 Bioleaching

Bioleaching is a bio-hydrometallurgical technique to extract metals using microbial activities, generally of Fe(II)- and S⁰-oxidizing acidophiles, from minerals. The technique is often used for recovery of various metals (e.g., copper; Yang et al 2009, zinc; Deveci et al 2004, lead; Liao et al 2004, arsenic; Zhang et al 2007, nickel; Le et al 2006, molybdenum; Nasernejad et al 1999, gold; Curreli et al 1997, silver; Frias et al 2002 and cobalt; Olson et al 1990).

1.3.2 Advantages and disadvantages of bioleaching techniques

Bioleaching technique shows several advantages in terms of economic and environmental impacts compared with pyrometallurgical approach and even with chemical leaching technique. Pyrometallurgical approaches can process ores containing target metals in high-grade, but not those in low-grade. Hydrometallurgical approaches are, in contrast, generally used for processing of low-grade ores. The main reason for the fact is economic efficiency: Operation, management and maintenance costs in hydrometallurgical approaches are generally low compared with those in pyrometallurgy.

In general, large amount of chemical compounds is necessary to facilitate leaching reaction in a chemical leaching technique. However, bioleaching technique requires much less amount of the chemicals owing to microbial activities that induce “recycling” of the chemicals. For example, ferric iron, one of the most common oxidizing agents, can be re-generated by microbial Fe(II)-oxidizing capability, resulting in ferric iron “recycling” in bioleaching technique.

As for disadvantages of bioleaching technique, there are a few points to be improved. One of them is its treatment efficiency: Reaction time in bioleaching system is relatively longer than that in chemical leaching, which is often caused by a lag phase of microbial growth. Given that microbial activity is more sensitive to operational conditions (e.g., temperatures, degree of oxygen supply) compared with chemical leaching technique and that it is hard to make microbial activities stop once they started, maintenance of optimal operational conditions may be sometimes difficult.

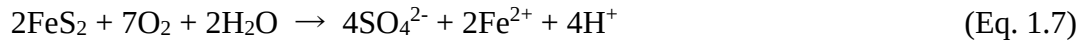
1.3.3 Practical bioleaching operations

Today, bioleaching of copper sulfide ores is widely operated in Chile, with more than 85,000 tons of ores processed per day (Brierley 1999). Major operations of copper recovery are conducted by Sociedad Contractual Minera El Abra and Codelco Division Radimiro Tomic in Chile, with 225,000 and 180,000 tons of metallic copper production per year, respectively. GeoBiotics, LLC has also developed several techniques for bioleaching and also biooxidation of sulfide concentrates (e.g., GEOCOAT® and GEOLEACH™). GEOCOAT® offers a unique approach to microbial leaching by combining the low capital and operating costs of heap leaching with the high recovery. The technology is applicable to the biooxidation of refractory sulfide gold concentrates, and to sulfidic copper, nickel, cobalt, zinc, and polymetallic base metal concentrates. The other one, GEOLEACH™, is a proprietary whole-ore bioleaching technology used to recover copper and other base metals from primary and secondary sulfide ores. In its process, ores are agglomerated, placed in a heap, and the heap is then operated in a similar way as a heap in GEOCOAT® process. The leachate containing target metals is further processed for specific metal recovery. The GEOLEACH™ technology maximizes heat conservation via control of aeration and irrigation rates (available online at <http://www.geobiotics.com>; 18th August, 2014).

Bioleaching technique has recently been successfully applied to secondary copper sulfide ores such as chalcocite and covellite. However, there are only a few cases that low-grade primary copper sulfide ores (e.g., chalcopyrite and bornite) are processed in industrial-scaled bioleaching operations (Watling 2006). Therefore, further studies on improvement of chalcopyrite bioleaching, efficiency should be conducted worldwide.

1.4 Bioleaching of sulfide minerals

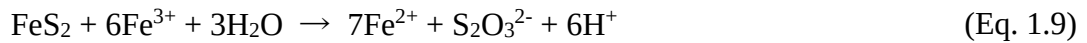
In sulfide mineral bioleaching system, Fe(II)- and S⁰-oxidizing microorganisms are generally involved as important keys. In order to understand a mechanism of sulfides bioleaching, bioleaching of pyrite (FeS₂) is discussed as follows (Fowler et al 1999b, Yahya et al 2002, Rodríguez et al 2003). As a starting reaction of pyrite bioleaching, pyrite is slightly oxidized by dissolved oxygen under acidic conditions as follows;



The produced Fe(II) is then oxidized by Fe(II)-oxidizing microorganisms as follows;



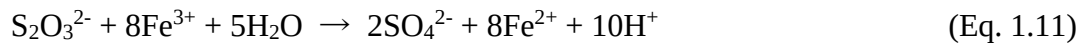
The microbially-produced Fe(III) which is strong oxidizing agent, oxidizes pyrite to thiosulfate as follows;



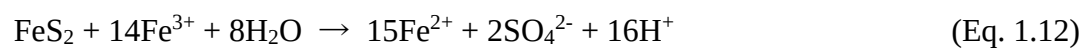
Another important microbial reaction occurs. Thiosulfate is also oxidized to sulfate by S⁰-oxidizing microorganisms as follows;



Oxidation of thiosulfate also occurs by Fe(III) as follows;



The abiotically/microbially-produced Fe(II) at equation Eq. 1.9 or 1.11, can be re-oxidized to Fe(III) by Fe(II)-oxidizing microorganisms (Eq. 1.8). Therefore, pyrite oxidation reaction can be activated owing to the Fe cycle. The overall reaction of pyrite bioleaching is described as follows;



Large amount of protons produced, results in acidification of bioleaching media. On the basis of the chemical/microbiological reactions, it is well-known that the presence of both Fe(II)- and S⁰-oxidizing microorganisms activates the pyrite bioleaching.

1.5 Chalcopyrite bioleaching

1.5.1 Copper

Copper (Cu) is one of the most essential elements with a number of industrial usages (e.g., electrical wires, semi-conductors, data transmission lines, communications tools, currencies and marine applications). Copper wires are the most efficient among those made of other metals (except for of silver), due to its high electro-conductivity. Consumption of copper has been increasing in developing countries, such as China and India. China had consumed approximately 40% of world's copper consumption in 2012, indicating that China has developed dramatically since around 2000. Despite of the fact above, China is not the biggest copper producer in the world: Twenty-nine percent of the world's copper production (i.e., approximately 6 million tons in 2012) was produced by Chile. Other main copper producers in 2012 included Peru, USA, China, Australia, Zambia, Indonesia, Congo and Brazil.

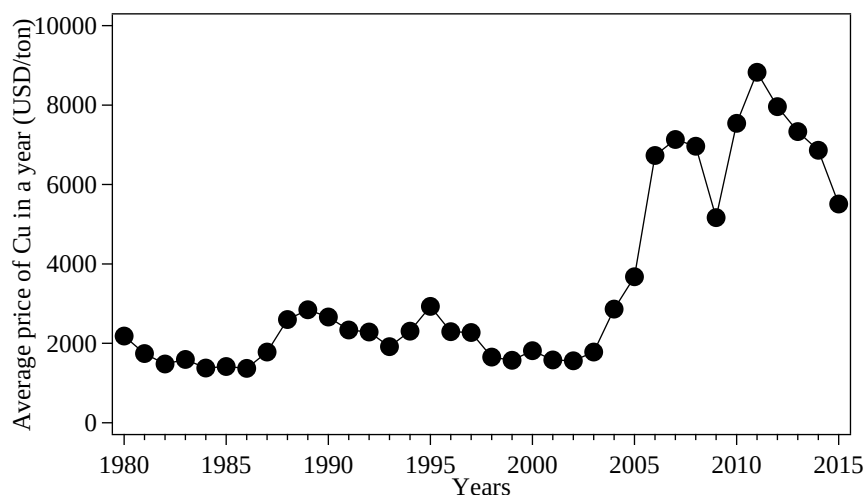


Fig. 1.2 Changes in Cu price from 1980 to 2015, available online at http://ecodb.net/pcp/imf_usd_pcopp.html (6th November, 2016).

In general, porphyry copper deposits account for approximately 65% of the world's copper reserves mainly as copper oxides; cuprite (Cu_2O) and malachite ($\text{Cu}_2\text{CO}_3(\text{OH})_2$), secondary copper sulfides; chalcocite (Cu_2S) and covellite (CuS), and primary copper sulfides; chalcopyrite (CuFeS_2) and bornite (Cu_5FeS_4). Today, copper is mainly produced from secondary sulfide ores (e.g., chalcocite and covellite), rather than from primary sulfides (e.g., chalcopyrite and bornite), since the latters are

generally more refractory and lower in grade. From 2000 to 2012, the price of copper has been rising. In 2000, copper was traded for 1.8 USD/ton. However, in 2012, the price has become 8.0 USD/ton over four times to the price in 2000. The reasons include; (i) increasing consumption of copper, especially in developing countries, (ii) declining grades of copper ores, and (iii) lack of new large copper deposits. Although its price has gradually decreased after 2012 to 2015, its price is likely stable due to its importance in the future. Therefore, development of Cu extraction techniques from even low grade refractory ores, such as chalcopyrite, is necessary to realize continuous copper supply to the industrialized society.

1.5.2 Chalcopyrite

Chalcopyrite (CuFeS_2) is a type primary copper sulfides and is composed of copper, iron and sulfur in the tetragonal system with golden yellow color (Burdick et al 1917). As described above, copper consumption worldwide has grown continuously. Therefore, it is required to extract copper from not only secondary copper sulfides but also from primary copper sulfides (e.g., chalcopyrite). Recently, a number of studies available to chalcopyrite bio/chemical leaching have been reported since chalcopyrite is the most abundant copper-containing mineral in the lithosphere. In general, copper extraction processes from copper concentrates have been operated either pyrometallurgically or hydrometallurgically, followed by electrolytic refining processes to finally obtain copper as a metal (99.99%). Since the pyrometallurgical process including thermal treatment requires high grade copper ores due to its high operating cost, lower grade copper ores are not applicable to the process. On the other hand, hydrometallurgical process including aqueous chemistry and has advantages over the former, in terms of copper extraction from low grade ores. Therefore, hydrometallurgical approach for copper extraction from chalcopyrite has been actively studied.

1.5.3 Microbial systems used for chalcopyrite bioleaching

1.5.3.1 The role of microorganisms

In chalcopyrite bioleaching system, utilization of both Fe(II)- and S⁰-oxidizing acidophilic microorganisms are preferable. The former acidophiles play an important role of re-generation of Fe(III) from Fe(II). The latter acidophiles is also important to prevent accumulation of elemental sulfur onto chalcopyrite surface, causing to inhibitory effect of its leaching reaction: The S⁰-oxidizing acidophiles oxidize elemental sulfur to sulfate, resulting in acidification of bioleaching media (Dopson et al 1999, Fowler et al 1999a).

1.5.3.2 Representative microorganisms used for chalcopyrite bioleaching

Acidithiobacillus (At.) (formerly Thiobacillus) ferrooxidans

At. ferrooxidans is an autotrophic, acidophilic and moderately thermophilic bacterium, capable of growing on ferrous iron or sulfur compounds as electron donor. In general, it grows optimally at pH 1.5-2.5 at 45-50°C. Therefore, it is commonly found in acid mine drainage containing various metal ions at low pHs. Although it basically grows under aerobic conditions, it is able to grow under anaerobic conditions using ferric iron as electron acceptor, instead of oxygen (Ohmura et al 2002).

At. (formerly Thiobacillus) thiooxidans

At. thiooxidans is an autotrophic, acidophilic and mesophilic bacterium, capable of growing on elemental sulfur as a primary electron donor under strictly aerobic conditions. The optimum temperature and pH for its growth are 28-30°C and less than 4.0, respectively. Additionally, it grows also on thiosulfate (S₂O₃²⁻) and tetrathionate (S₄O₆²⁻), but not hydrogen sulfide and sulfides (Waksman et al 1922).

At. (formerly Thiobacillus) caldus

At. caldus is a mixotrophic, acidophilic and moderately thermophilic bacterium, capable of growing on elemental sulfur and tetrathionate, but not on ferrous iron. The optimum temperature and pH for active growth of *A. caldus* are 45°C and 2.0-2.5, respectively (Hallberg et al, 1994).

Acidianus brierleyi

Acidianus brierleyi is a thermophilic and extremely acidophilic archaeon. It autotrophically grows on elemental sulfur and ferrous iron as electron donor, at optimum pH of 1.5-2.0 and temperature of 70°C. It is able to also grow under anaerobic conditions by reducing sulfur (Seeger et al 1986).

Leptospirillum (L.) ferrooxidans

L. ferrooxidans is an obligately autotrophic, mesophilic and acidophilic bacterium, capable of growing ferrous iron, but incapable of oxidizing elemental sulfur and RISCs (reduced inorganic sulfur compounds) (Hippe 2000). The optimum temperature and pH for its growth are 25-35°C and 2.5-3.0, respectively. Therefore, *L. ferrooxidans* is often applied to bioleaching of sulfide minerals with *At. ferrooxidans* or *At. thiooxidans* (Bosecker 1997).

L. ferriphilum

L. ferriphilum is an obligately autotrophic, moderately thermophilic and acidophilic bacterium. It grows aerobically at optimum pH of 1.5 and temperature of 43°C. It obtains energy for growth by oxidizing ferrous iron as electron donor. It was reported to grow on also pyrite, but not tetrathionate (Okibe et al 2003).

Sulfobacillus (Sb.) thermosulfidooxidans

Sb. thermosulfidooxidans is a moderately thermophilic and acidophilic bacterium, capable of growing either heterotrophically or autotrophically on ferrous iron, tetrathionate, elemental sulfur, thiosulfate, and sulfide minerals (Pina et al 2010, Xia et al 2010). It is able to grow in a wide range of temperature and pH; at 20-60°C and pH 1.5-5.5, respectively. Optimum temperature and pH are 50-55°C and 1.9-2.4, respectively.

Sulfolobus (S.) metallicus

S. metallicus is an extremely thermophilic, obligately autotrophic, and acidophilic archaeon. It grows aerobically at 50-75°C and pH 1.0-4.5 on elemental sulfur and sulfide (Huber et al 1991, Gautier et al 2008, Morales et al 2011). Additionally, its

growth via oxidation of pyrite and ferrous iron was also observed (Norris et al 2000).

1.5.4 Factors affecting bioleaching efficiency

1.5.4.1 Supply of oxygen and carbon dioxide

Microorganisms in chalcopyrite bioleaching systems are basically aerobic. In general, bioleaching operations are often conducted in large-scaled bioreactors or heaps. In the case of bioleaching in large-scaled bioreactors, the reactor inside is usually stirred and aerated by air. In the case of bioleaching in heaps, it is also necessary to maintain heap inside aerobically by supplying air using tubes. In the absence of oxygen supply, microbial activity is de-activated, leading to failure of bioleaching operation.

Carbon dioxide is also necessary as carbon source for growth of autotrophic microorganisms. Therefore, a lack of carbon dioxide results in inactive the growth of the microorganisms, such as *At. ferrooxidans* and *L. ferrooxidans*, leading to less efficiency of chalcopyrite bioleaching process. However, excess concentration of carbon dioxide was reported to show inhibitory effect on microbial growth: Dissolved carbon dioxide at concentrations of more than 10 mg/L was found to inhibit growth of *A. ferrooxidans* on pyrite-arsenopyrite-pyrrothite ores (Nagpal et al 1993). It was reported that optimal concentration of carbon dioxide for effective bioleaching operation was in a range of 7 to 17% (Kock et al 2004).

1.5.4.2 Temperature

In order to activate microbial activities, temperature of bioleaching operation is necessary to be maintained in the optimal range for its microbial system, since every microorganism has its own optimum temperature. As for mesophiles, the growth rate was reported to reduce by approximately 50%, for every 6°C temperature decrease in the range between 25 to 2°C (Norris 1990). Several research groups compared metal extraction efficiency between mesophiles and thermophiles. The efficiency of sulfide minerals bioleaching by thermophiles was higher than that by mesophiles (Barr et al, 1992, Jordan et al, 1993, Clark et al, 1996, Norris et al, 1996). In chalcopyrite bioleaching at mesophilic temperature (30°C), copper recovery was reported to be 22

and 42% when using pure culture of *At. ferrooxidans* and mixed culture of *At. ferrooxidans*, *At. thiooxidans*, and *At. caldus*, respectively, within 30 days (Bevilaqua et al 2002, Fu et al 2008). On the other hand, in chalcopyrite bioleaching at thermophilic temperature (70°C), copper recovery was reported to be 85% using pure culture of *Sulfolobus* sp. within 30 days (Gomez et al 1999). These facts obtained at different temperatures indicate that chalcopyrite is more effectively bioleached at 70°C rather than at 30°C. Other research group compared chalcopyrite bioleaching efficiencies using thermophiles (mainly *Sulfolobus* spp.) at 35°C and mesophiles (mainly *Acidithiobacillus* spp. and *Leptospirillum* spp.) and 68°C: Cu recovery in the former and the latter marked 56 and 9.5%, respectively, within 40 days (Rodríguez et al 2003). However, bioleaching operation using thermophiles at higher temperatures would take much more cost compared with that at lower temperatures (e.g., natural temperatures). This is, in fact, one of the main disadvantages of utilization of thermophiles to chalcopyrite bioleaching technique.

1.5.4.3 pH

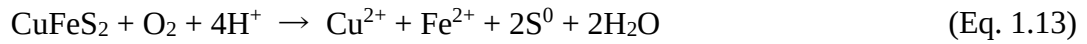
As well as temperature, solution pH of chalcopyrite bioleaching is also important factor in order to achieve higher copper extraction efficiency. In general, bioleaching is operated at lower pHs since Fe(II)- and S⁰-oxidizing microorganisms are often acidophilic. Considering only chemical reaction of chalcopyrite leaching, leaching efficiency can be improved at lower pHs (Vilcáez et al 2009). The reasons for the fact might be (i) chalcopyrite dissolution reaction consumes protons, and (ii) secondary ferric iron precipitation (e.g., goethite, jarosite) occurs when pH increases over around 2-3. Dorado et al (2012) reported that chalcopyrite chemical leaching efficiency was found to be enhanced at lower pHs: Decrease in 2.5 pH units implied an increase in the efficiency of 35%. Vilcáez et al (2008) however reported that optimum conditions (e.g., pH, temperature) for bioleaching should be investigated depending on microbial systems used. As described above, given that Since chalcopyrite consumes protons for its dissolution, and that increase in pH causes ferric precipitation resulting in passivation effect of chalcopyrite dissolution, maintenance of pHs at low level (e.g., 1.5 to 2.0) critically affects its leaching efficiency. Therefore, the most important key

of chalcopyrite bioleaching system is utilization of S^0 -oxidizing acidophiles capable of producing protons through its S^0 oxidation (Waksman et al, 1922, Segerer et al, 1986, Larsson et al, 1990, Hallberg et al, 1994, Pina et al, 2010, Xia et al, 2010).

1.5.5 Chalcopyrite bioleaching mechanisms

1.5.5.1 Chalcopyrite bioleaching mechanism at higher redox potential levels

According to a conventional understanding of chalcopyrite bioleaching, its mechanism has been recognized as oxidation by ferric iron (Watling 2006). As a starting reaction of chalcopyrite bioleaching, it is slightly oxidized by dissolved oxygen under acidic conditions as follows;



The reaction speed of the chemical reaction above is low. In order to leach chalcopyrite, strong oxidizing agent, Fe(III) is necessary as follows;



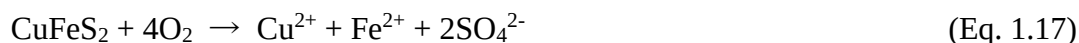
The produced Fe(II) is then oxidized by Fe(II)-oxidizing microorganisms as follows;



The microbially-produced Fe(III) oxidize chalcopyrite again, resulting in continuous oxidation of chalcopyrite by Fe(III) owing to Fe(II)-oxidizing microorganisms (Third et al 2000). In chalcopyrite bioleaching, S^0 accumulated onto the chalcopyrite surface, which declines its leaching efficiency. Therefore, S^0 -oxidizing microorganisms play a role to increase yield of copper extraction as follows;



The net reaction of chalcopyrite bioleaching is described as follows;



The bioleaching mechanism of chalcopyrite is described below.

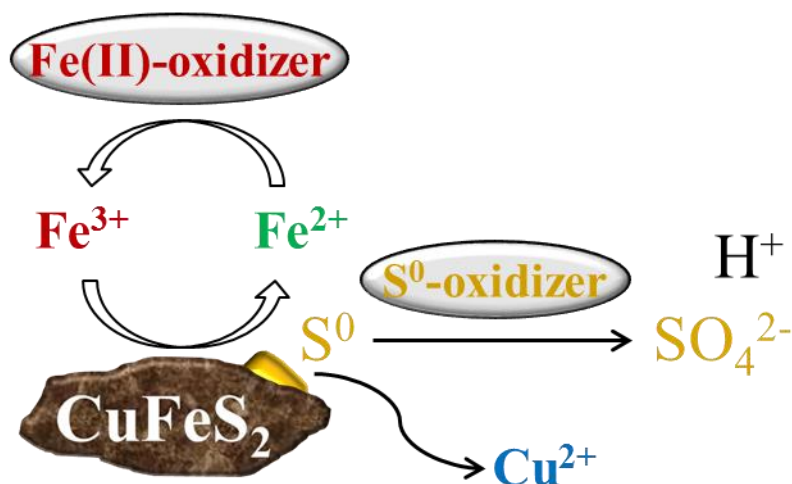


Fig. 1.3 Scheme of chalcopyrite bioleaching system at higher redox level.

In this mechanism of chalcopyrite bioleaching, oxidant Fe(III) is the key. Therefore, dominant species of soluble Fe is required to be Fe(III) form, but not Fe(II), which means higher solution redox potential level is preferable for chalcopyrite bioleaching through oxidation by Fe(III).

Chalcopyrite bioleaching efficiency using different microbial systems, initial pHs, temperatures, and pulp densities, are reviewed below.

Table 1.4 Summary of chalcopyrite bioleaching reviews at higher redox potential level at thermophilic temperatures (50-85°C).

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Batch	Ref.
<i>Acidians brierleyi</i>	1.5	85	10	14	1	29% as Cu	100 mL (△ 200)	Vilcáez 2015
<i>S. metallicus</i>	1.5	85	36	14	1	29% as Cu	100 mL (△ 200)	Vilcáez 2015
<i>M. sedula</i>	1.5	85	20	14	1	29% as Cu	100 mL (△ 200)	Vilcáez 2015
<i>S. metallicus</i>	1.5	80	95	14	1	83	100 mL (△ 250)	Vilcáez et al 2008
<i>Acidians brierleyi</i>	1.5	80	94	12	1	83	100 mL (△ 250)	Vilcáez et al 2008
<i>M. sedula</i>	2	80	93	14	1	83	100 mL (△ 250)	Vilcáez et al 2008
<i>Acidians brierleyi</i>	1.5	80	92	14	1	83	100 mL (△ 250)	Vilcáez et al 2008
<i>Acidians brierleyi</i>	2.5	80	87	14	1	83	100 mL (△ 250)	Vilcáez et al 2008
<i>M. sedula</i>	1.5	80	82	14	1	83	100 mL (△ 250)	Vilcáez et al 2008
<i>S. metallicus</i>	2	80	78	14	1	83	100 mL (△ 250)	Vilcáez et al 2008

continued

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Batch	Ref.
<i>M. sedula</i>	2.5	80	45	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	2	75	100	11	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	1.5	75	94	12	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	1.5	75	93	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	2.5	75	93	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>S. metallicus</i>	1.5	75	92	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>M. sedula</i>	1.5	75	89	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>M. sedula</i>	2.5	75	66	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>S. metallicus</i>	2	75	55	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	2	75	54	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008

continued

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Batch	Ref.
<i>M. sedula</i>	2	75	50	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>S. metallicus</i>	2.5	75	15	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	1.5	70	100	10	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	2	70	98	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	1.5	70	91	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>S. metallicus</i>	1.5	70	85	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>S. metallicus</i>	2	70	82	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>M. sedula</i>	1.5	70	69	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	2.5	70	59	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>M. sedula</i>	2.5	70	55	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008

continued

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Batch	Ref.
<i>S. metallicus</i>	2.5	70	47	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>M. sedula</i>	2	70	42	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	2	70	38	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Sulfolobus BC</i>	1.5	68	92	9	2	9.2		Romano et al 2001
A mixed culture of thermophiles (mainly <i>Sulfolobus</i> spp.)	1.5	68	56	40	5	55	100 mL	Rodríguez et al 2003
<i>Acidianus brierleyi</i>	1.5	65	100	11	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	2	65	94	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	1.5	65	81	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>M. sedula</i>	1.5	65	71	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008

continued

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Batch	Ref.
<i>S. metallicus</i>	1.5	65	60	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>S. metallicus</i>	2	65	60	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>M. sedula</i>	2	65	53	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>M. sedula</i>	2.5	65	29	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	2	65	26	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>S. metallicus</i>	2.5	65	26	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	2.5	65	16	14	1	83	100 mL (△ 250)	Vilc á ez et al 2008
<i>Acidianus brierleyi</i>	1.5	65	95	14	1	83	100 mL (△ 250)	Vilc á ez et al 2009
<i>S. metallicus</i>	1	65	94	14	1	83	100 mL (△ 250)	Vilc á ez et al 2009
<i>Acidianus brierleyi</i>	1.5	65	75	14	1	21% as Cu	100 mL (△ 200)	Vilc á ez et al 2015

continued

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Batch	Ref.
<i>Acidianus brierleyi</i>	1.5	65	95	14	1	29% as Cu	100 mL (△ 200)	Vilcáez 2015
<i>Acidianus brierleyi</i>	1.5	65	90	14	1	29% as Cu	100 mL (△ 200)	Vilcáez 2015
<i>S. metallicus</i>	1.5	65	60	14	1	29% as Cu	100 mL (△ 200)	Vilcáez 2015
<i>M. sedula</i>	1.5	65	58	14	1	29% as Cu	100 mL (△ 200)	Vilcáez 2015
<i>Acidianus brierleyi</i>	2	65	75	14	2	35% as Cu	200 mL (△ 500)	Zhu et al 2011
<i>M. sedula</i>	2	65	73	14	2	35% as Cu	200 mL (△ 500)	Zhu et al 2011
<i>A. manzaensis</i>	2	65	74	14	2	35% as Cu	200 mL (△ 500)	Zhu et al 2011
<i>S. metallicus</i>	2	65	32	14	2	35% as Cu	200 mL (△ 500)	Zhu et al 2011
A mixed culture of <i>Acidianus brierleyi</i> , <i>M. sedula</i> , <i>A. manzaensis</i> , and <i>S. metallicus</i>	2	65	89	14	2	35% as Cu	200 mL (△ 500)	Zhu et al 2011
<i>Sb. thermosulfidooxidans</i>	2	53	42	20	1	94	100 mL (△ 250)	Xia et al 2010

Table 1.5 Summary of chalcopyrite bioleaching reviews at higher redox potential level at moderately thermophilic temperatures (40-45°C).

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Batch	Ref.
A mixed culture of moderate thermophiles	1.5	45	51	17	2	9.2		Romano et al 2001
<i>At. caldus</i>	2	45	21	7	1	90	100 mL (△ 250)	Chen et al 2008
<i>L. ferriphilum</i> and <i>At. caldus</i> (2:1)	1.6	40	54	32	2	95	100 mL (△ 250)	Fu et al 2008
<i>L. ferriphilum</i> and <i>At. caldus</i> (1:2)	1.6	40	49	32	2	95	100 mL (△ 250)	Fu et al 2008
<i>L. ferriphilum</i> and <i>At. caldus</i> (1:1)	1.6	40	44	32	2	95	100 mL (△ 250)	Fu et al 2008
<i>L. ferriphilum</i>	1.6	40	40	32	2	95	100 mL (△ 250)	Fu et al 2008
<i>At. caldus</i>	1.6	40	11	32	2	95	100 mL (△ 250)	Fu et al 2008
<i>At. ferrooxidans</i>	2.8	40	5.9	26	5	81	(△500)	Karimi et al 2010

Table 1.6 Summary of chalcopyrite bioleaching reviews at higher redox potential level at mesophilic temperatures (30-37°C).

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Batch	Ref.
A mixed culture of mesophilic acidophiles	1.5	37	59	16	4	88	100 mL (△ 500)	Third et al 2002
A mixed culture of mesophilic acidophiles	1.5	37	22	13	4	88	100 mL (△ 500)	Third et al 2000
A mixed culture of mesophilic acidophiles	1.5	37	13	13	4	88	100 mL (△ 500)	Third et al 2000
A mixed culture of mesophiles (<i>At. ferrooxidans</i> , <i>At. thiooxidans</i> and <i>L. ferrooxidans</i>)	1.8	35	50	20	2	9.2		Romano et al 2001
A mixed culture of mesophiles (mainly <i>Acidithiobacillus</i> spp. and <i>Leptospirillum</i> spp.)	1.7	35	10	40	5	55	100 mL	Rodríguez et al 2003
<i>At. ferrooxidans</i>	2	30	30	75	-	96	△250	Zhang et al 2008
<i>At. ferrooxidans</i>	2	30	46	32	2	95	100 mL (△ 250)	Fu et al 2008
<i>At. ferrooxidans</i> and <i>At. caldus</i>	2	30	41	32	2	95	100 mL (△ 250)	Fu et al 2008

continued

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Batch	Ref.
<i>At. ferrooxidans</i> and <i>At. thiooxidans</i>	2	30	39	32	2	95	100 mL (△ 250)	Fu et al 2008
<i>At. ferrooxidans</i> , <i>At. thiooxidans</i> , and <i>At. caldus</i>	2	30	36	32	2	95	100 mL (△ 250)	Fu et al 2008
<i>At. thiooxidans</i>	2	30	9.6	32	2	95	100 mL (△ 250)	Fu et al 2008
<i>At. ferrooxidans</i>	2.8	30	21	26	5	81	(△500)	Karimi et al 2010
<i>At. ferrooxidans</i>	2	30	27	7	1	90	100 mL (△ 250)	Chen et al 2008
<i>At. ferrooxidans</i> strain LR	1.6	30	57	56	2.5	72	150 mL (△ 250)	Bevilaqua et al 2002
<i>At. ferrooxidans</i> strain LR	1.6	30	27	56	2.5	72	150 mL (△ 250)	Bevilaqua et al 2002
<i>At. ferrooxidans</i> , <i>At. thiooxidans</i> and <i>L. ferrooxidans</i>	1.6	30	62	24	1	45	100 mL (△ 250)	Akcil et al 2007
<i>At. thiooxidans</i>	2.1	30	8	24	5	45	100 mL (△ 250)	Akcil et al 2007

continued

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Batch	Ref.
<i>At. ferrooxidans</i> , <i>At. thiooxidans</i> and <i>L. ferrooxidans</i>	1.6	30	56	24	2	45	100 mL (△ 250)	Akcil et al 2007
<i>At. ferrooxidans</i>	1.6	30	50	24	1	45	100 mL (△ 250)	Akcil et al 2007
<i>At. ferrooxidans</i> , <i>At. thiooxidans</i> and <i>L. ferrooxidans</i>	1.6	30	50	24	3	45	100 mL (△ 250)	Akcil et al 2007
<i>At. ferrooxidans</i>	1.6	30	47	24	2	45	100 mL (△ 250)	Akcil et al 2007
<i>At. ferrooxidans</i>	1.6	30	47	24	2	45	100 mL (△ 250)	Akcil et al 2007
<i>At. ferrooxidans</i> , <i>At. thiooxidans</i> and <i>L. ferrooxidans</i>	1.6	30	45	24	5	45	100 mL (△ 250)	Akcil et al 2007
<i>At. ferrooxidans</i>	1.6	30	43	24	3	45	100 mL (△ 250)	Akcil et al 2007
<i>L. ferrooxidans</i>	1.6	30	39	24	1	45	100 mL (△ 250)	Akcil et al 2007

continued

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Batch	Ref.
<i>At. ferrooxidans</i>	1.6	30	38	24	5	45	100 mL (△ 250)	Akcil et al 2007
<i>L. ferrooxidans</i>	1.6	30	34	24	2	45	100 mL (△ 250)	Akcil et al 2007
<i>L. ferrooxidans</i>	1.6	30	31	24	3	45	100 mL (△ 250)	Akcil et al 2007
<i>L. ferrooxidans</i>	1.6	30	26	24	5	45	100 mL (△ 250)	Akcil et al 2007
<i>At. thiooxidans</i>	2.1	30	13	24	1	45	100 mL (△ 250)	Akcil et al 2007
<i>At. thiooxidans</i>	2.1	30	11	24	2	45	100 mL (△ 250)	Akcil et al 2007
<i>At. thiooxidans</i>	2.1	30	9	24	3	45	100 mL (△ 250)	Akcil et al 2007

Chalcopyrite bioleaching has been carried out using lots of microbial system under various conditions. The bioleaching at thermophilic temperatures are found to often be carried out using *Acidianus brierleyi*, *S. metallicus*, and *M. sedula* as microbial systems, resulting in relatively higher Cu recovery, in some cases reaching to 100%. The distribution of Cu recovery depends on initial pHs, temperatures, pulp densities, and chalcopyrite grades. As for chalcopyrite bioleaching at moderate temperatures, utilization of *At. ferrooxidans*, *At. thiooxidans*, and *L. ferriphilum* has been commonly studied. Since leaching temperature critically affects chalcopyrite leaching efficiency, those Cu recoveries at moderate temperatures were lower than those at thermophilic temperatures: Maximum Cu recovery at moderate temperatures was reported to be 54% within 32 days using a mixed culture of *L. ferriphilum* and *At. caldus* in a volume ratio of inoculum, 2:1 (at pH 1.6, 40°C; Fu et al 2008). At mesophilic temperature (37°C), relatively effective Cu recovery (59%) in chalcopyrite bioleaching system was marked using a mixed culture of mesophilic acidophiles at initial pH of 1.5 (Third et al 2002), however, Cu recovery values at mesophilic and moderately thermophilic temperatures were found to be far lower than those at thermophilic temperature. As for initial pH of leaching solution, 1.5 to 2 seem to be common range to be set at. The highest and the lowest among the reviews above are 2.8 and 1, respectively. In fact, determination of initial pH values depends on an optimum pH range of microbial systems used, since use of S^0 -oxidizing acidophiles results in culture acidification, leading to maintenance of pH level below initial pHs in successful chalcopyrite bioleaching system.

1.5.5.2 Chalcopyrite bioleaching mechanism at lower redox potential levels

Among a number of papers available on chalcopyrite ($CuFeS_2$) bioleaching, maximization of Cu extraction has been investigated with a conventional understanding of its mechanism (Zhang et al 2008, Vilcáez et al 2008, Fu et al 2008, Gautier et al 2008, Karimi et al 2010): Chalcopyrite is oxidized by oxidant Fe(III) as shown in Eq. 1.18. Microbial Fe(II)-oxidizing activity plays an important role in re-oxidation of Fe(II) to Fe(III), resulting in continuous supply of the oxidant (Eq. 1.19). S^0 -oxidizing microorganisms are often used in order to prevent accumulation of elemental sulfur on chalcopyrite surface (Eq. 1.20).



In this mechanism, higher solution redox potential level is required via microbial Fe(II) oxidation in order to continuously oxidize chalcopyrite by Fe(III). Formation of jarosite, however, often occurs at high solution redox potential level, causing passivation of chalcopyrite surface, followed by declining in Cu extraction rate (Córdoba et al. 2008, 2009).

Enhancement of Cu recovery in chalcopyrite bioleaching system at relatively low redox potential level, rather than at higher redox level, was reported: Third et al (2000) reported that addition of bacterial inoculum (a mixed culture of mesophilic acidophilic bacteria) in different ratios (1 10, and 50%) to bioleaching media resulted in different Cu extraction rates: Higher Cu extraction rate was reported when bacterial inoculum was added in a ratio of 10%, which was caused by the fact that its solution redox potential level was maintained relatively low (around 600 mV vs. SHE). The research group also reported improvement in Cu recovery by redox potential control via limitation of oxygen supply (Third et al 2002): Cu extraction rate was more efficient with a redox potential setpoint of 380 mV (vs. Ag/AgCl) through oxygen supply limitation, rather than that with continuous supply of oxygen (leading to redox potentials over 600 mV vs. Ag/AgCl). Promotion of Cu recovery from chalcopyrite concentrate in chemical leaching system using ferrous-dominant sulfate solution was also reported (Córdoba et al 2008, Vilcáez et al 2009).

Chalcopyrite bioleaching tests that redox potentials were electrochemically controlled, have been also studied using bioreactors or bench-scale piloting facilities. Gericke et al (2010) concluded that Cu extraction from chalcopyrite concentrate was maximized when redox potentials were electrochemically controlled at around 420 mV (vs. Ag/AgCl) in bioleaching system at 45°C. Ahmadi et al (2010) also reported that Cu extraction from chalcopyrite concentrate (10% pulp density) in bioleaching system at 50°C reached approximately 90% by 22 days with electrochemical control of redox

potentials at 400 to 450 mV (vs. Ag/AgCl), while chalcopyrite bioleaching that redox potentials were not controlled resulted in 65% by 22 days. The research group reported that 77% chalcopyrite dissolution was achieved with the pulp density of 20% via electrochemical bioleaching at 400 to 430 mV (vs. Ag/AgCl) (Ahmadi et al 2011). Both research groups mentioned that less-activated pyrite oxidation and less amount of jarosite formation can be recognized as key advantages when redox potentials are electrochemically controlled at around 425-430 mV (vs. Ag/AgCl) in electrochemical bioleaching of chalcopyrite concentrate.

The mechanism of chalcopyrite bio/chemical leaching at relatively low redox potential level was found to be explained by involvement of an intermediate chalcocite (Cu_2S). Hiroyoshi et al (2000, 2001, 2004, 2008) reported that dissolution of chalcopyrite proceeds through formation of chalcocite as an intermediate: The formation (Eq. 1.21) and oxidation (Eq. 1.22) of chalcocite was found to promote chalcopyrite dissolution as follows;

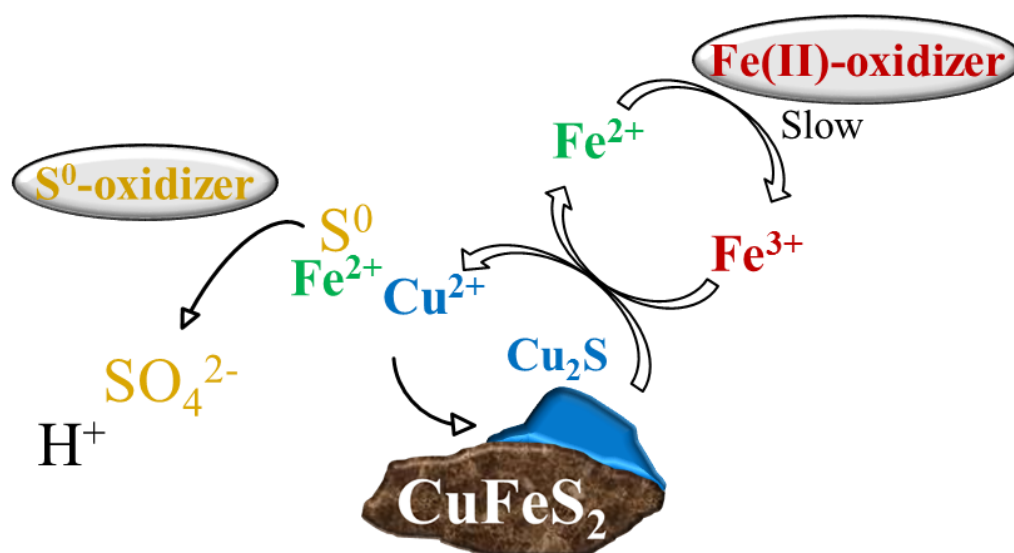
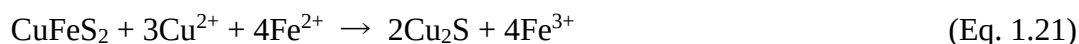


Fig. 1.4 Scheme of chalcopyrite bioleaching system at lower redox level.

Owing to S^0 -oxidizing microorganisms, elemental sulfur produced at chalcocite oxidation, is oxidized to sulfate, resulting in generation of protons. The key of the leaching mechanisms is the fact that Fe(II), but not Fe(III), should be dominant species in soluble Fe, in order to form chalcocite as an intermediate. Therefore, microbial oxidation of Fe(II) to Fe(III) is preferable to be “less-active”.

From a viewpoint of thermodynamics, reaction equation of chalcocite formation and its critical potential, E_c , are described below (at 1 atm);



$$E_c (V) = E_c^0 + \frac{RT}{4F} \ln \frac{(a_{Cu^{2+}})^3}{a_{Fe^{2+}}} \quad (\text{Eq. 1.24})$$

, where R, T, F, and a_i are gas constant (J/Kmol), temperature (K), faraday constant (C/mol), and the activities of species i . E_c^0 indicates the standard redox potential (V) of Eq. 1.20. Reaction equation of oxidation of chalcocite and its oxidation potential, E_{ox} , are described below (at 1 atm);



$$E_{ox} (V) = E_{ox}^0 + \frac{RT}{4F} \ln (a_{Cu^{2+}})^2 \quad (\text{Eq. 1.26})$$

, where E_{ox}^0 indicates the standard redox potential (V) of Eq. 1.22. The reaction model was found to be strongly affected by the solution redox potential levels (Hiroyoshi et al 2001, 2004, 2008): Enhanced Cu extraction from chalcopyrite in the model can be thermodynamically described at the solution redox potentials of;

$$E_{ox} < E < E_c \quad (\text{Eq. 1.27})$$

Scheme of the leaching mechanism of chalcopyrite through formation of chalcocite as an intermediate and its oxidation is shown in Fig. 1.5.

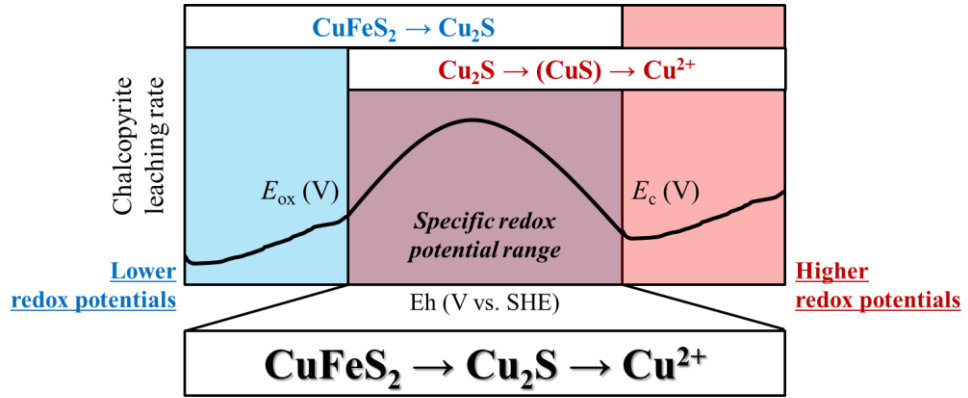


Fig. 1.5 Redox potential dependence of chalcopyrite leaching through intermediate, chalcocite.

Okamoto et al. (2004) defined “ E_{normal} ” to evaluate optimal solution redox potential levels in the chalcopyrite leaching system, and proposed the maximization of the chalcopyrite dissolution at the E_{normal} value of 0.43.

$$E_{\text{normal}} = (E - E_{\text{ox}}) / (E_c - E_{\text{ox}}) \quad (\text{Eq. 1.28})$$

$$E_c \text{ (V)} = 0.72 + 0.059 \log \frac{(a_{\text{Cu}^{2+}})^{0.75}}{(a_{\text{Fe}^{2+}})^{0.25}} \quad (\text{Eq. 1.29})$$

$$E_{\text{ox}} \text{ (V)} = 0.60 + 0.059 \log (a_{\text{Cu}^{2+}})^{0.5} \quad (\text{Eq. 1.30})$$

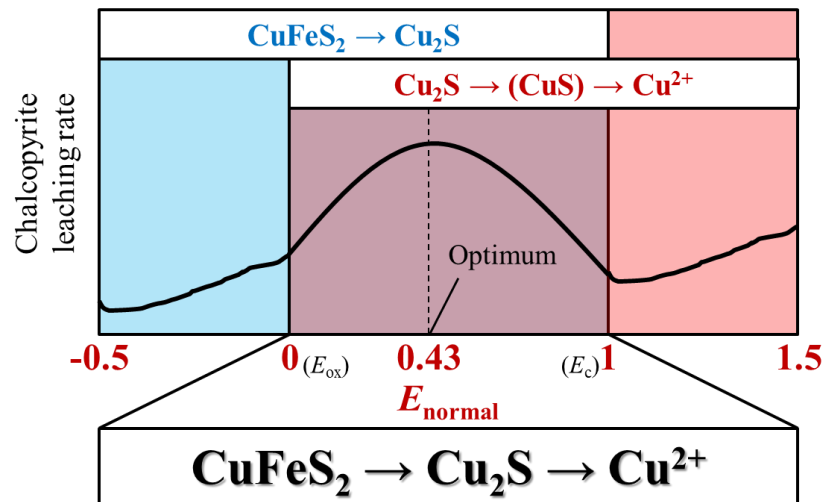


Fig. 1.6 E_{normal} dependence of chalcopyrite leaching through intermediate, chalcocite.

The information about redox dependence of chalcopyrite dissolution in bioleaching system that redox potential is not controlled electrochemically is yet highly limited, and evaluation of the chalcopyrite leaching behaviors using E_{normal} values was only reported by electrochemical and chemical leaching experiments, but not by bioleaching test. Therefore, chalcopyrite bioleaching behaviors was evaluated through E_{normal} values in this study. Furthermore, solution redox potential level was microbiologically controlled using different degree of Fe(II)-oxidizing capabilities deriving from different Fe(II)-oxidizers.

Chalcopyrite bioleaching efficiency obtained at relatively lower redox potential levels (redox controlled) are reviewed in Table 1.7.

In the bioleaching mechanism of chalcopyrite at lower redox potential level, Fe(II)-oxidizing microorganisms play an important role of slow supply of Fe(III), in order to increase solution redox potentials over E_{ox} , but below E_c . As is discussed above, Third et al (2002) had managed to control redox potentials by suppression of growth of Fe(II)-oxidizing microorganisms via limitation of oxygen supply, resulting in observation of significant improvement in Cu extraction efficiency at relatively lower redox potential level (around 600 mV vs. SHE, probable between E_{ox} and E_c) than at higher redox level (around 800 mV vs. SHE, probable corresponding to over E_c). The research group mentioned that utilization of S^0 -oxidizing microorganisms only could be an attractive alternative approach to succeed redox-controlled bioleaching. However, utilization of Fe(II)-oxidizing microorganisms showing relatively weaker Fe(II)-oxidizing capability by which solution redox potential level is maintained between E_{ox} and E_c , rather than dismissing microbial Fe(II) oxidation, would possibly succeed in maintenance of redox potential level between E_{ox} and E_c , for effective Cu recovery from chalcopyrite concentrate. Therefore, in chapter 8, solution redox potential level was managed to be microbiologically controlled using different degree of Fe(II)-oxidizing capabilities deriving from different Fe(II)-oxidizers.

Table 1.7 Summary of chalcopyrite bioleaching reviews at lower redox potential level at mesophilic temperatures (37°C).

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Batch	Ref.
A mixed culture of mesophilic acidophiles	1.5	37	70	16	4	88	100 mL (Δ 500)	Third et al 2001
A mixed culture of mesophilic acidophiles	1.5	37	50	13	10	88	100 mL (Δ 500)	Third et al 2000
A mixed culture of moderate thermophiles	1.5	37	50	13	10	88	101 mL (Δ 500)	Third et al 2001
A mixed culture <i>At. ferrooxidans</i> , <i>At. thiooxidans</i> and <i>L. ferrooxidans</i>)	1.8	35	62	22	10	44	Electro-bioreactor (Eh controlled at 400-450 mV vs. Ag/AgCl)	Ahmadi et al 2010
A mixed culture of mesophilic acidophiles	1.8	50	90	22	10	44	Electro-bioreactor (Eh controlled at 400-450 mV vs. Ag/AgCl)	Ahmadi et al 2010
A mixed culture of mesophilic acidophiles	1.5	50	75	10	20	44	Electro-bioreactor (Eh controlled at 400-430 mV vs. Ag/AgCl)	Ahmadi et al 2011
A mixed culture of mesophilic acidophiles	1.5	50	66	10	20	44	Electro-bioreactor (Eh controlled at 440-480 mV vs. Ag/AgCl)	Ahmadi et al 2011

1.5.6 Addition of silver catalyst to chalcopyrite bioleaching systems

Chalcopyrite chemical-bio leaching efficiency was reported to be facilitated by addition of silver catalyst in the form of Ag_2S , Ag_2SO_4 or AgCl (Mier et al 1995, Sato et al 2000, Hiroyoshi et al 2002, López-Juárez et al 2006, Muñoz et al 2007, Deng et al 2009, Feng et al 2013). The proposed leaching mechanism is discussed as below;



The Ag cycle promotes chalcopyrite dissolution. Due to the both chemical reactions, large amount of Fe(II) is produced, resulting in decrease in solution redox potential level. Since Fe(III) is one of the important keys for oxidation of silver sulfide, higher solution redox potential in leaching solution contributes further increase in Cu extraction rate from chalcopyrite. Therefore, utilization of Fe(II)-oxidizing microorganisms is meaningful, as well as of S^0 -oxidizing microorganisms responsible to oxidation of elemental sulfur to sulfate, as was shown in Eq. 1.15 and 1.16, respectively. Scheme of bioleaching mechanism of chalcopyrite with the addition of Ag catalyst is described in Fig. 1.7. Mechanisms of silver catalyzed-chalcopyrite leaching are described in “introduction” in chapter 7 in detailed.

Given that Cu recovery from chalcopyrite using mesophilic and moderately thermophilic acidophiles has been limited to around 50%, and that that using thermophilic acidophiles has reached over 90%, chalcopyrite bioleaching using the former acidophiles is worth being studied. The addition of silver catalyst is one of the choices to enhance its Cu recovery in bioleaching systems at mesophilic and moderate temperatures.

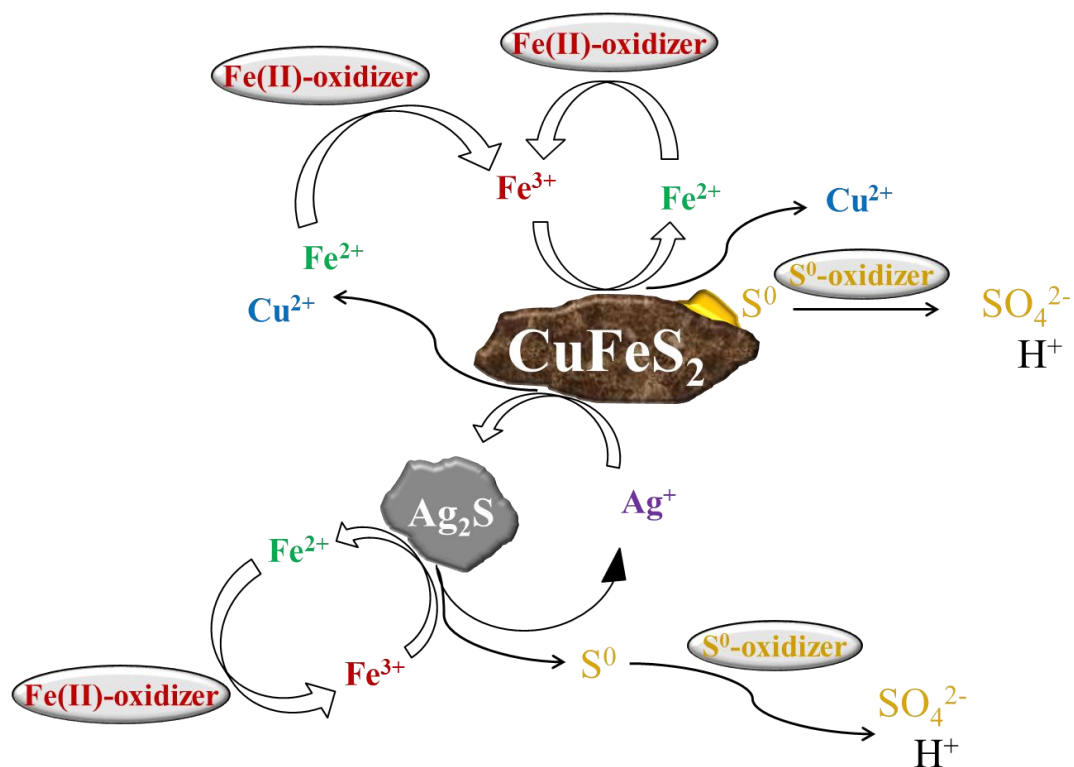


Fig. 1.7 Scheme of bioleaching mechanism of chalcopyrite with the addition of Ag catalyst.

Table 1.8 Summary of silver-catalyzed chalcocopyrite bioleaching reviews.

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Ag addition	Batch	Ref.
A mixed culture of <i>At. ferrooxidans</i> strain CUMT-1 & <i>Acidithiobacillus</i> sp. strain ZJJN	2	30	52	20	1	3	50 mg/L	100 mL (△ 500)	Feng et al 2013
A mixed culture of <i>At. ferrooxidans</i> strain CUMT-1 & <i>Acidithiobacillus</i> sp. strain ZJJN	2	30	48	20	1	3	10 mg/L	100 mL (△ 500)	Feng et al 2013
A mixed culture of <i>At. ferrooxidans</i> strain CUMT-1 & <i>Acidithiobacillus</i> sp. strain ZJJN	2	30	50	20	1	3	2 mg/L	100 mL (△ 500)	Feng et al 2013
A mixed culture of <i>At. ferrooxidans</i> strain CUMT-1 & <i>Acidithiobacillus</i> sp. strain ZJJN	2	30	38	20	1	3	0.5 mg/L	100 mL (△ 500)	Feng et al 2013

continued

Microbial systems	Initial pH	Temp. (°C)	Cu leached (%)	Leaching periods (day)	Pulp density (%)	CuFeS ₂ grade (%)	Ag addition	Batch	Ref.
<i>At. ferrooxidans</i>	1.3	25	65	22	2	59	1000 mg/L	200 mL (△ 250)	Sato et al 2000
<i>At. ferrooxidans</i>	1.3	25	62	22	2	59	500 mg/L	200 mL (△ 250)	Sato et al 2000
<i>At. ferrooxidans</i>	1.3	25	58	22	2	59	100 mg/L	200 mL (△ 250)	Sato et al 2000
A mixed culture of <i>Thiobacilli</i> and <i>Leptospirilli</i>	2	35	93	15	10	10	66 mg/L	75 mL (△ 250)	Muñoz et al 2007

1.6 Objectives of the thesis

This work is divided into two parts; fundamental studies and application studies. In the former part (**chapter 3 and 4**), search for useful iron-reducing acidophiles and characterization of representative *Sulfolobus* strains regarding iron redox abilities were focused on. The objectives in the fundamental studies were to contribute to better understandings of geochemical Fe cycles in highly acidic environments and to show possible applicability of the microbiological iron redox transformation by strains tested in this study to mineral processing systems.

In the latter part (application studies), potential utility of the microbiological iron redox transformation to mineral processing techniques was studied, including reductive bioleaching of Indonesian nickel-containing limonite ore and chalcopyrite concentrate, silver catalyzed-chalcopyrite bioleaching, microbiologically redox potential controlled-chalcopyrite bioleaching. **In chapter 5**, dissolution of limonite ore for nickel recovery via reductive bioleaching using microbial Fe(III) reduction was investigated. Since the above approach to ferric mineral showed some effectiveness, the approach was utilized to chalcopyrite dissolution for copper recovery. **In chapter 6**, applicability of the reductive bioleaching to chalcopyrite, refractory primary copper sulfide, was investigated. Although several research groups have observed improvement in copper recovery in the presence of silver catalyst among a number of papers available on chalcopyrite bioleaching, its detailed mechanism is yet to be elucidated. Therefore, the objectives **in chapter 7** were set as follows; (i) to evaluate effect of silver on the chalcopyrite bioleaching and (ii) to investigate its detailed mechanism. **In chapter 8**, redox potential dependence of chalcopyrite dissolution in the bioleaching system was investigated, especially focusing on the microbiological redox potential control during the bioleaching tests. Therefore, the objectives are set as follows; (i) to evaluate the potential utility of the microbiological redox potential control for maximization of copper dissolution from chalcopyrite concentrates, and (ii) to investigate its detailed mechanism from the viewpoint of electrochemistry and leaching kinetics.

Overall, objective of this work was to investigate fundamental microbiological iron redox transformation and its possible applicability to mineral processing techniques.

1.7 Structure of the thesis

This work is divided into 9 chapters.

Chapter 1 represents the background of importance of microbiological iron redox transformation and its utilization to mineral processing techniques. Previous studies related to the present study are reviewed and discussed in this chapter. In addition, objectives of this work are presented.

Chapter 2 shows preparations of microbial culture media, maintenances of microorganisms, and pre-treatments of mineral concentrates, used in this work. Additionally, common experimental procedures and analytical methods used in this work, are described.

Chapters 3 and 4 present fundamental studies focusing on microbiological iron redox transformation.

In chapter 3, microbial community structure of Blood Pond Hell hot spring in Japan was investigated in order to search useful metal-reducing acidophiles, applicable to mineral processing and bioremediation of heavy metal-polluted environments. Sequence of the 16Sr RNA gene (1381 base pair) of an isolates was analyzed. The isolate was characterized in terms of its iron redox ability and reduction capability of hexavalent chromium, highly toxic heavy metal often contaminated in heavy metal-polluted environments such as acid mine drainage.

In chapter 4, iron redox transformation by the thermo-acidophilic archaea, *Sulfolobus* species, was investigated. Five representative type strains of *Sulfolobus* (*S.*) spp.; *S. shibatae* B12^T, *S. tokodaii* 7^T, *S. acidocaldarius* 98-3^T, *S. solfataricus* P1^T, and *S. metallicus* Kra23^T, and *Sulfolobus* sp. GA1, isolated and characterized in chapter 3, were compared their iron reducing/oxidizing abilities. Capabilities of hexavalent chromium reduction and the following chromium immobilization were also studied with the *Sulfolobus* strains in this chapter.

Chapters 5, 6, 7, and 8 represent application studies to mineral processing.

In chapter 5, improvement of nickel recovery via reductive bioleaching of Indonesian Ni-limonite ore using iron-reducing acidophiles was investigated. The chapter is divided into 4 sections. Section 1 describes screening of useful iron-reducing acidophiles to the reductive bioleaching tests of the limonite ore, based on their effectiveness in soluble Fe(III) reduction and subsequent dissolution of chemically

synthesized goethite. In section 2, useful iron-reducing acidophiles selected in section 1 were utilized to the reductive bioleaching of acid washed-limonite ore, and its dissolution behaviors were discussed. In section 3, citrate washed-limonite ore was used in order to prevent excess increase in solution pH due to calcium contents still remained in acid washed-limonite ore. Improvement of Ni recovery from the citrate washed-limonite ore was investigated through single, double, and triple steps of the reductive bioleaching test.

In chapter 6, reductive bioleaching was utilized to dissolution of refractory primary copper sulfide, chalcopyrite.

In chapter 7, effect of silver sulfate (section 1) and silver sulfide (section 2) on chalcopyrite bioleaching was investigated. Various mixed cultures of iron- and sulfur-oxidizing acidophiles were tested in order to improve copper recovery in silver catalyzed-chalcopyrite bioleaching system. Its detailed mechanism was discussed and factors to be improved were suggested.

In chapter 8, potential utility of the microbiological redox potential control in chalcopyrite bioleaching system was investigated. On the basis of the fact that chalcocite is involved in chalcopyrite dissolution as an intermediate, effect of the microbiological redox potential control on chalcopyrite bioleaching efficiency was discussed. The key of the experiment is that degree of iron-oxidizing capability of iron-oxidizers are different to target different levels of solution redox potential. Relationship between the chalcopyrite dissolution and the E_{normal} level was discussed in this chapter.

Chapter 9 represents summary of the main conclusions based on the results and discussion in this study, and the recommendation for future work.

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Chapter 2

Methodology

2.1 Culture medium and chemical reagents used in this study

2.1.1 Heterotrophic basal salts (HBS)

Solution composition of HBS (50×)

22.5 g/L (NH₄)₂SO₄

2.5 g/L KCl

2.5 g/L KH₂PO₄

25 g/L MgSO₄·7H₂O

0.7 g/L Ca(NO₃)₂·4H₂O

7.1 g/L Na₂SO₄

Solubilized into distilled water, stored in sterilized bottle at 4°C. To make 1 L of HBS (1×) media, 20 mL of HBS stock (50×) was mixed with 980 mL of distilled water and sterilized by autoclave (120°C, 20 min).

2.1.2 Chemical reagents used in this study

1M Ferric iron stock solution

Fe₂(SO₄)₃·nH₂O (n = 5-7; Wako pure chemicals) was solubilized into acidic distilled water (pH 1.5 with H₂SO₄), and Fe(III) concentration was measured by *o*-phenanthroline method using ascorbic acid as reducing agent. Based on the Fe(III) concentration, the Fe(III) stock was diluted with acidic distilled water until its Fe concentration becomes 1 M. Filtrated (0.02 µm) and stored in sterilized bottle at 4°C.

1M Ferrous iron stock solution

FeSO₄·7H₂O (Wako pure chemicals) was solubilized into acidic distilled water (pH 1.5 with H₂SO₄), filtrated (0.02 µm), and stored in sterilized bottle at 4°C.

20 mM Hexavalent chromium stock solution

Na₂CrO₄·4H₂O (Wako pure chemicals) was solubilized into acidic distilled water (pH 2.0 with H₂SO₄), filtrated (0.02 µm), and stored in sterilized bottle at 4°C.

20 mM Trivalent chromium stock solution

Cr(NO₃)₃·9H₂O (Wako pure chemicals) was solubilized into acidic distilled water (pH 2.0 with H₂SO₄), filtrated (0.02 µm), and stored in sterilized bottle at 4°C.

5000 mg/L Ag(I) stock solution

Ag₂SO₄ (Wako pure chemicals) was solubilized into acidic distilled water (pH 1.5 with H₂SO₄), filtrated (0.02 µm), and stored in sterilized bottle at 4°C.

1 M Glucose stock solution

Glucose (Wako pure chemicals) was solubilized into acidic distilled water (pH 2.0 with H₂SO₄), filtrated (0.02 µm), and stored in sterilized bottle at 4°C.

1 M Glucose stock solution

Fructose (Wako pure chemicals) was solubilized into acidic distilled water (pH 2.0 with H₂SO₄), filtrated (0.02 µm), and stored in sterilized bottle at 4°C.

10% (w/v) Yeast extract stock solution

Yeast extract (Sigma-Aldrich) was solubilized into acidic distilled water (pH 2.0 with H₂SO₄), filtrated (0.02 µm), and stored in sterilized bottle at 4°C.

Trace elements (1000×) stock solution

The following chemicals (Wako pure chemicals) were solubilized into distilled water, filtrated (0.02 µm), and stored in sterilized bottle at 4°C.

10 mg/L ZnSO₄·7H₂O

1 mg/L CuSO₄·5H₂O

1.09 mg/L MnSO₄·5H₂O

1 mg/L CoSO₄·7H₂O

0.39 mg/L Cr₂(SO₄)₃·7H₂O

0.6 mg/L H₃BO₃

0.5 mg/L Na₂MoO₄·2H₂O

0.1 mg/L NaVO₃

1 mg/L NiSO₄·6H₂O

0.51 mg/L Na₂SeO₄

0.1 mg/L Na₂WO₄·2H₂O

Sterilized elemental sulfur powder

Powder of elemental sulfur (Wako pure chemicals) was sterilized in oven (100°C, overnight, twice) and stored in sterilized bottle at 4°C.

2.2 Microorganisms used in this study**2.2.1 Culture maintenances**

Table 2.1 Microbial culture maintenances.

Microorganisms	pH	Temp.	e ⁻ donor	Others
<i>S. shibatae</i> B12 (NR044677)	2.0	70°C	10 mM Glucose	0.01% (w/v) y.e.
<i>S. tokodaii</i> 7 (NR074348)	2.0	70°C	10 mM Glucose	0.01% (w/v) y.e.
<i>Sulfolobus</i> sp. GA1	2.0	70°C	10 mM Glucose	0.01% (w/v) y.e.
<i>S. acidocaldarius</i> 98-3 (NR043400)	3.0	70°C	10 mM Glucose	0.01% (w/v) y.e.
<i>S. solfataricus</i> P1 (NR119198)	3.0	70°C	10 mM Glucose	0.01% (w/v) y.e.
<i>S. metallicus</i> Kra23 (NR119199)	2.0	70°C	0.01% (w/v) S ⁰	Trace element stock
<i>Ali. acidocaldarius</i> subsp. <i>acidocaldarius</i> 104-IA (CP001727)	3.0	60°C	10 mM Glucose	0.01% (w/v) y.e.
<i>Ali. acidiphilus</i> TA-67 (AB076660)	3.0	45°C	10 mM Glucose	0.01% (w/v) y.e.
<i>Ali. acidoterrestris</i> GD3B (AB042057)	3.0	45°C	10 mM Glucose	0.01% (w/v) y.e.
<i>Sb. acidophilus</i> YTF1	2.0	45°C	10 mM Glucose	0.1-1.0% (w/v) y.e.
<i>Acidiplasma</i> sp. Fv-Ap	1.5	45°C	10 mM Fe(II)	0.01% (w/v) y.e.
<i>Sb. sibiricus</i> N1 (AY079150)	1.5	45°C	10 mM Fe(II)	0.01% (w/v) y.e.

Microorganisms	pH	Temp.	e ⁻ donor	Others
<i>Am. ferrooxidans</i> ICP (CP001631)	1.5	45°C	10 mM Fe(II)	0.01% (w/v) y.e.
<i>At. caldus</i> KU (Z29975)	2.0	45°C	0.01% (w/v) S ⁰	Trace elements stock
<i>Fp. acidiphilum</i> strain Y (AJ224936)	1.5	45°C	10 mM Fe(II)	Trace elements stock
<i>Acidiphilium</i> sp. SJH	3.0	30°C	10 mM Glucose	0.01% (w/v) y.e.
<i>Ac. aromatica</i> PFBC	3.0	30°C	10 mM Fructose	0.01% (w/v) y.e.
<i>Ferrimicrobium</i> sp. T23	1.5	30°C	10 mM Fe(II)	0.01% (w/v) y.e.

All the cultures were maintained in 300 mL Erlenmeyer flask containing 100 mL HBS media (pH adjusted as the above) supplemented with the electron donors and yeast extract or trace elements, as is described in Table 2.1. The flasks were maintained at the temperatures (Table 2.1) on an orbital rotary shaker at 100-150 rpm.

2.2.2 Sub-culturing

All the sub-culturing was carried out using 500 mL Erlenmeyer flask containing 200 mL HBS media: Media composition was same as that of the stock culture.

2.3 Minerals used in this study

2.3.1 Indonesian limonite ore

Acid washed limonite:

The Indonesian limonite ore was washed with 1 M HNO₃ (5 min, at RT), deionized water (5 min, at RT) and finally with 100% ethanol (5 min, at RT) in order to remove residual metals and surface oxide film on the ore. After that, the concentrate was freeze-dried overnight.

Citrate washed limonite:

The raw Indonesian limonite ore was washed with 1 M citric acid (pulp density of 10%(w/v), stirred at 500 rpm for 7 days at RT) in order to solubilize serpentine ((Ni²⁺, Mg²⁺, Fe²⁺)₃Si₂O₅(OH)₄), but not goethite. Then, the ore was washed with 100%

ethanol twice (5 min, at RT), and subsequently freeze-dried overnight.

Table 2.2 Mineralogical composition of raw, acid washed, and citrate washed Indonesian limonite according to XRF analysis.

mass % Elements	Raw limonite	Acid washed limonite	Citrate washed limonite
Fe	38.6	40.8	41.3
Si	10.2	9.3	12.3
Mg	5.1	4.3	3.0
Al	2.8	3.2	1.9
Ni	2.8	3.0	2.1
Ca	2.0	1.1	0.5
Cr	1.5	1.6	1.5
Mn	0.43	0.46	0.31
S	0.028	0.036	< 10 µg/g
Zn	0.052	0.054	0.037
V	0.026	0.026	0.022
K	0.035	< 1 µg/g	< 1 µg/g
Sr	0.0047	0.0038	< 0.1 µg/g

2.3.2 Chemically synthesized goethite

Procedure of synthesis:

1. Add 50 g of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ into 500 mL of pure water (pH 1.2 with H_2SO_4) and stirred at 600 rpm overnight.
2. Add 2 M KOH until solution pH of 1.2. Dried at 60°C for 5 days.
3. Washed with 1 M HNO_3 (5 min, at RT), deionized water (5 min, at RT) and finally with 100% ethanol (5 min, at RT), and then freeze-dried.

X-ray diffraction pattern of the synthesized goethite is shown in Fig. 2.1.

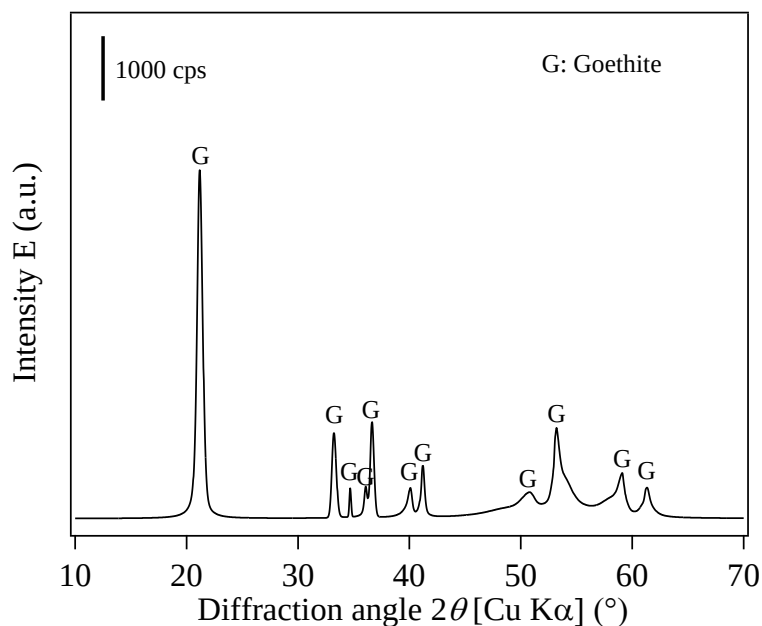


Fig. 2.1 X-ray diffraction patterns of goethite chemically synthesized. “G” indicates the peak patterns corresponding to goethite.

2.3.3 Concentrates of copper sulfides

Chalcopyrite (CuFeS₂)

Chalcopyrite concentrate (produced in Chili) was kindly provided by JX Nippon Mining & Metals. For all the chalcopyrite bioleaching experiment, acid washed chalcopyrite concentrate was used.

Pyrite (CuFeS₂)

Pyrite concentrate (produced in Chili) was purchased by IWAMOTO mineral. Acid washed pyrite concentrate was used for bioleaching experiment.

Chalcocite (Cu₂S)

Chalcocite concentrate (produced in Leonard mine, Butte, Montana, U.S.A.) was purchased by IWAMOTO mineral. Acid washed chalcocite concentrate was used for XAFS experiment.

Covellite (CuS)

Covellite concentrate (produced in Masutomi mine, Yamanashi, Japan) was

purchased by IWAMOTO mineral. Acid washed covellite concentrate was used for XAFS experiment.

Acid wash treatment

The concentrates were washed with 1 M HNO₃ (5 min, 25°C), deionized water (5 min, 25°C) and finally with 100% ethanol (5 min, 25°C) in order to remove residual metals and surface oxide film on the concentrate. After that, the concentrate was freeze-dried overnight.

Table 2.3 Mineralogical composition of raw chalcopyrite, acid washed chalcopyrite, acid washed chalcocite, acid washed covellite, and acid washed pyrite, according to ICP analysis after acid (aqua regia) dissolution using microwave treatment.

mass % Elements	Raw chalcopyrite	Acid washed chalcopyrite	Acid washed chalcocite	Acid washed covellite	Acid washed pyrite
Cu	32.5	32.7	84.8	30.0	0.5
Fe	26.2	28.8	0.7	0	42
S	35.2	37.1	22.6	15.3	48.3
Cu/S	0.92	0.88	3.75	1.96	0.01

2.4 Experimental conditions

Aerobic conditions

Aerobic conditions were established using air permeable silicon caps for Erlenmeyer flasks.

Micro-aerobic conditions

Micro-aerobic conditions were established using falcon tubes (15 or 50 mL) or serum bottles (50 or 100 mL serum bottles with rubber stoppers): The oxygen to establish micro aerobic conditions therefore came from the solutions used to make the medium which were not purged with N₂. When serum bottles were used, liquid samples were taken using syringes with needles in order to avoid contamination of oxygen.

Anaerobic conditions

Anaerobic conditions were established using serum bottles (20, 50 or 100 mL serum bottles with rubber stoppers). Liquid media and all the chemical reagent stock solution used were N₂-purged (dissolved oxygen < 0.1 mg/L). Liquid samples were taken using syringes with needles in order to avoid contamination of oxygen.

2.5 Sampling procedures

2.5.1 Liquid samples

Liquid samples taken from the experimental cultures after compensation of water evaporated with pure water, and then used for cell counting using microscope, filtered using 0.20- μ m cartridge filters, and used for measurements of metal concentrations, pH values and solution redox potential values.

2.5.2 Solid residues

At the end of the experiments, solid residues were collected by filtration using vacuum pump, and freeze-dried overnight.

2.6 pH and solution redox potential values measurements

Solution pH and redox potential values (Ag/AgCl reference electrode) were measured using pH-Eh meter (MM-60R, TOADKK). The measured solution redox potential values were automatically converted to values vs. SHE as follows;

$$“E \text{ vs. SHE}” = “E \text{ vs. Ag/AgCl}” + 206 - 0.7 (“\text{Solution Temp.}” - 25) \quad (\text{Eq. 2.1})$$

E: Solution redox potentials (V)

2.7 Spectrophotometer

2.7.1 O-phenanthroline method

O-phenanthroline method was used in this study as a Fe(II) assay. The procedures are described below (Caldwell and Adams 1946).

1. Add 30 μL of 1 M HCl to some wells of 96-well measuring plate.
2. Add 30 μL of liquid samples to the wells. Note that all samples were centrifuged (12,000 rpm, 8 min) using Bio Shaker G·BR-200 (TAITEC), and subsequently diluted (e.g., $\times 10$, etc.) using 1 M HCl if needed.
3. Add 30 μL of ascorbic acid solution to the wells in the case of total soluble Fe measurements. Note that ascorbic acid solution was made on all such occasions since the chemical is unstable in solution (one spoon of ascorbic acid powder (Wako pure chemicals) solubilized into 5 mL of distilled water).
4. Give 5 min to react Fe(III) ions and ascorbic acid.
5. Add 30 μL of 5 mM *o*-phenanthroline solution (solubilized in distilled water) to the wells in order to form $[\text{Fe}(\text{phenanthroline})_3]^{2+}$ complex.
6. Add 30 μL of 2 M sodium acetate solution (solubilized in distilled water) to the wells.
7. Add distilled water until total volume of 300 μL .
8. Give 10 min to get stable.
9. Measure absorbance at 510 nm using spectrophotometer (Multiskan Go, Thermo Scientific).

A standard curve used is shown in Fig. 2.2. Note that calibration curve was redrawn every time new chemical reagents were made.

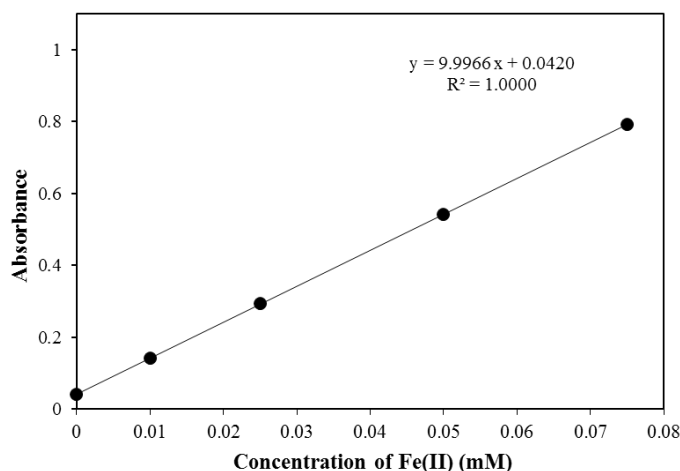


Fig. 2.2 Standard curve of Fe(II) concentration using *o*-phenanthroline method.

$$\text{Concentration of Fe(II) (mM)} = (\text{Absorbance} - 0.0420)/9.9966$$

$$R^2 = 1.0000$$

2.7.2 Diphenyl carbazide method

Diphenyl carbazide method was used in this study as a Cr(VI) assay. The procedures are described below (Noroozifar and Khorasani-Motlagh 2003).

1. Add 30 μL of 1.8 M H_2SO_4 to some wells of 96-well measuring plate.
2. Add 30 μL of liquid samples to the wells. Note that all samples were centrifuged (12,000 rpm, 8 min) using Minispin (Eppendorf), and subsequently diluted (e.g., $\times 10$, etc.) using 1 M H_2SO_4 if needed.
3. Add 30 μL of mixed reagent of diphenyl carbazide solution (1% (w/v) 1, 5 diphenyl carbon hydrazide and 60% (v/v) acetone solubilized in distilled water) to the wells.
4. Add distilled water until total volume of 300 μL .
5. Give 10 min to get stable.
6. Measure absorbance at 530 nm using spectrophotometer (Multiskan Go, Thermo Scientific).

A standard curve used is shown in Fig. 2.3. Note that calibration curve was redrawn every time new chemical reagents were made.

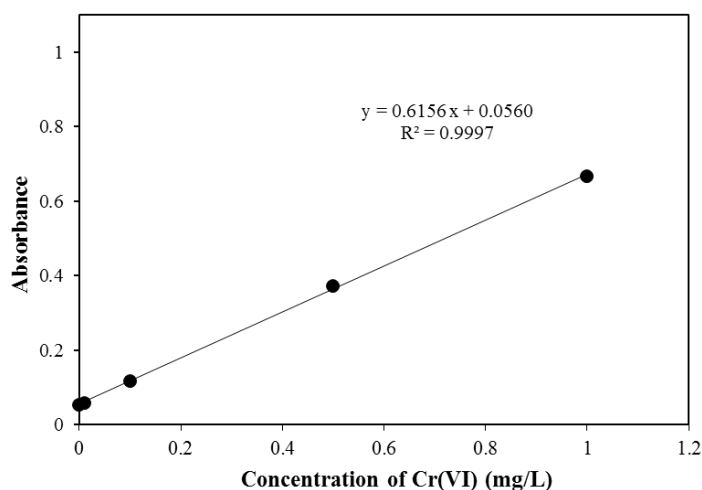


Fig. 2.3 Standard curve of Cr(VI) concentration using diphenyl carbazide method.

$$\text{Concentration of Cr(VI) (mg/L)} = (\text{Absorbance} - 0.0560)/0.6156$$

$$R^2 = 0.9997$$

2.7.3 Bicinthonic acid method

Bicinthonic acid method was used in this study as a Cu(I) assay. The procedures are described below (Anwar et al 2000). Note that this method was used to determine concentrations of Cu(I) and total soluble Cu in 7.2.1 only.

1. Add 40 μL of liquid samples to small test tubes. Note that all samples were centrifuged (12,000 rpm, 8 min) using Minispin (Eppendorf), and subsequently diluted (e.g., $\times 10$, etc.) using 1 M HCl if needed.
2. Add 80 μL of ascorbic acid solution to the wells in the case of total soluble Cu measurements. Note that ascorbic acid solution was made on all such occasions since the chemical is unstable in solution (one spoon of ascorbic acid powder (Wako pure chemicals) solubilized into 5 mL of distilled water).
3. Add 400 μL of tartrate buffer (0.5 M sodium potassium tartrate solubilized in distilled water adjusted to pH 5.5 with HCl) and mix well.
4. If Fe(III) ion is present, add 200 μL of the phosphate buffer (mix 87.7 mL of 0.2 M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ with 13.3 mL of 0.2 M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) and centrifuge the sample before measuring the absorbance at 562 nm.
5. Add 40 μL of bicinthonic acid (dilute a commercially prepared 0.2% solution (i.e., from Sigma) to 0.1% with the tartrate buffer) and mix well.
6. Add distilled water until total volume of 1 mL.
7. Measure absorbance at 526 nm using spectrophotometer (Multiskan Go, Thermo Scientific).

A standard curve used is shown in Fig. 2.4. Note that calibration curve was redrawn every time new chemical reagents were made.

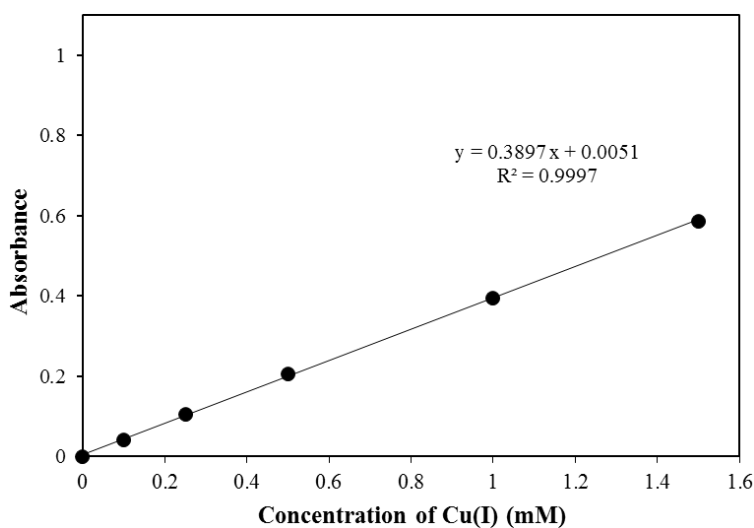


Fig. 2.4 Standard curve of Cu(I) concentration using bicinhoninic acid method.

$$\text{Concentration of Cu(I) (mM)} = (\text{Absorbance} - 0.0051)/0.3897$$

$$R^2 = 0.9997$$

2.8 Inductively coupled plasma-atomic emission spectrometry (ICP-AES)

Concentrations of total soluble metal ions (e.g., Fe, Cr, Cu, Ni, Ca, Mg, Al, Co) in acid solution samples were measured using ICP-AES (SEIKO Vista-MPX). After original liquid samples were taken from Erlenmeyer flasks, falcon tubes or serum bottles, they were filtered (0.20 μm) or centrifuged (12,000 rpm, 8 min) using Minispin (Eppendorf) in order to separate solution and solid phases, and subsequently diluted depending on estimated concentrations of target metal ions in them (e.g., $\times 25$, 100, 250, etc.) using 0.5 M HCl. All the measurements using ICP-AES were carried out in duplicates: The average values were used for the results.

Wavelengths measured for each metal are described as follows:

Fe: 238.204, 239.562, 259.939 nm (Wavelength of 238.204 nm was used for calculations of Fe concentrations.)

Cr: 267.716, 205.560, 283.563 nm (Wavelength of 267.716 nm was used for calculations of Cr concentrations.)

Cu: 327.393, 324.752, 224.700 nm (Wavelength of 327.393 nm was used for calculations of Cu concentrations.)

Ni: 231.604, 221.648, 232.003 nm (Wavelength of 231.604 nm was used for calculations of Ni concentrations.)

Ca: 317.933, 315.887, 393.366 nm (Wavelength of 317.933 nm was used for calculations of Ca concentrations.)

Mg: 285.213, 279.077, 280.271 nm (Wavelength of 285.213 nm was used for calculations of Mg concentrations.)

Al: 396.153, 308.215, 394.401 nm (Wavelength of 396.153 nm was used for calculations of Al concentrations.)

Co: 228.616, 238.892, 230.786 nm (Wavelength of 228.616 nm was used for calculations of Co concentrations.)

2.9 X-ray fluorescence (XRF)

ZSX Primus II, Rigaku

Elemental compositions of freeze-dried sediment samples collected from Chinoike-Jigoku hot spring, were analyzed using ZSX Primus II (Rigaku). Measurement were carried out with oxide mode (diameter irradiated X-ray was 10 mm) under vacuum conditions. The calculation was conducted using fundamental parameter (FP) method.

EDX-7000, Shimazu

Elemental compositions of freeze-dried Indonesian limonite ores were analyzed using EDX-7000 (Shimazu). Measurement were carried out with oxide mode (diameter irradiated X-ray was 5 mm) under He-purged conditions. The calculation was conducted using fundamental parameter (FP) method.

2.10 X-ray diffraction (XRD)

XRD measurement (Ultima IV, Rigaku) was performed with Cu-K α radiation as an X-ray source. The accelerating voltage and current were 40 kV and 40 mA, with a scanning speed of 2°/min and scanning step of 0.02°.

2.11 X-ray absorption near edge structure (XANES)

Tablet samples for XAFS measurement were prepared by using a tablet press machine at 10 MPa for 5 min. X-ray absorption spectra were collected on Kyushu University beam line (BL06) at Kyushu Synchrotron Light Research Center (SAGA-LS; 1.4 GeV storage ring with a circumference of 75.6 m). The measurements were conducted at the Cu K-edge (with the energy range from 8,650 to 10,500 eV) and Cr K-edge (with the energy range from 5,660 to 7,500 eV) and experimental data were collected in transmission mode. Energy selection was accomplished by a double crystal Si (1, 1, 1) monochromator. Intensities of incident and transmitted X-ray were recorded by using ionization chambers. Standard chemicals and samples were uniformly mixed with boron nitride (BN; Wako pure chemicals) in appropriate ratios for XAFS measurement.

2.12 X-ray photoelectron spectroscopy (XPS)

All XPS spectra were recorded with a ESCA 5800 (ULVAC-PHI) using Al K α line as an X-ray source under high vacuum conditions; 2×10^{-10} torr. The binding energy scale was referenced to the C 1s line of aliphatic carbon contamination, set at 284.6 eV. Processing of the XP-spectra was accomplished with Casa XPS software.

2.13 Scanning electron microscope (SEM)

SEM images were collected using VE-9800 (Keyence) and ULTRA55 (Zeiss) at accelerated voltage of 15-20 kV.

2.14 Electron probe micro analyzer (EPMA)

For preparation of EPMA specimens, the freeze-dried samples were embedded in resin and polished to determine the chemical composition of constituent minerals by Field Emission Electron Probe Micro Analyzer (FE-EPMA; JEOL JXA-8530F; 10 nA, 20 kV). Incident electron beam was focused to 1 mm in diameter and counting time was 5 seconds for each element (iteration of 5). The acquired X-ray intensities were

corrected by ZAF method to obtain element concentrations for a quantitative analysis. (Boekestein et al 1983).

2.15 Microwave treatment

Hydrothermal pre-treatment was carried out as follows: Teflon vessels containing a known amount of solid samples and 60% HNO₃ solution or aqua regia (12M HCL and 60% HNO₃ are mixed in a volume ratio 3:1) were placed in the microwave digestion system (Ethos Plus, Milestone) and heated to 230°C with 7°C /min increments, kept for 15 min at 230°C, and finally allowed to cool to room temperature.

2.16 Total organic carbon analyzer

Total organic carbon (TOC) content of sediment samples (from Chinoike-Jigoku hot spring) was analyzed using TOC-VCHS (Shimazu).

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Chapter 3

Investigation of microbial community structure of Blood Pond Hell hot spring in Japan and search for useful metal-reducing microorganisms

Abstract

Chinoike Jigoku (“Blood Pond Hell”) is located in the hot spring town of Beppu on the southern island of Kyushu in Japan, and is the site of a red-colored acidic geothermal pond. This study aimed to investigate the microbial population composition in this extremely acidic environment and to isolate/characterize acidophilic microorganism with metal-reducing ability. Initially, PCR (using bacteria- and archaea-specific primers) of environmental DNA samples detected the presence of bacteria, but not archaea. This was followed by random sequencing analysis, confirming the presence of wide bacterial diversity at the site (123 clones derived from 18 bacterial and 1 archaeal genera), including those closely related to known autotrophic and heterotrophic acidophiles (*Acidithiobacillus* sp., *Sulfobacillus* sp., *Alicyclobacillus* sp.). Nevertheless, successive culture enrichment with Fe(III) under micro-aerobic conditions led to isolation of an unknown archaeal organism, *Sulfolobus* sp. GA1 (with 99.7% 16S rRNA gene sequence identity with *S. shibatae* B12). Unlike many other known *Sulfolobus* spp., strain GA1 was shown to lack sulfur oxidation ability. Strain GA1 possessed only minor Fe(II) oxidation ability, but readily reduced Fe(III) during heterotrophic growth under micro-aerobic condition. Strain GA1 was capable of reducing highly toxic Cr(VI) to less toxic/soluble Cr(III), demonstrating its potential utility in bioremediation of toxic metal species.

3.1 Introduction

Japan lies on the “Ring of Fire” and its volcanic nature has endowed the country with a number of geothermal hot springs. These abundant extreme environments in the country are a precious resource for extremophiles (e.g., Atomi et al 2004, Suzuki et al 2002, Takayanagi et al 1996). Some of the many highly acidic hot springs in Japan are located on the southern island of Kyushu. These hot springs are a potential source of extremely acidophilic microorganisms with various applications in biohydrometallurgy.

Metal-polluted aqueous systems, including acid mine drainages (AMDs) and metal refinery process waters, are often highly acidic. When some toxic metal pollutants such as Cr(VI), V(V) and U(VI) are reduced to lower oxidation states, their toxicity and solubility can be decreased, facilitating their removal from the aqueous phase. Since Fe(III)-reducing microorganisms are often capable of reducing other metals (e.g., *Ac. aromatic*; Masaki et al 2015, *A. cryptum*; Cummings et al 2007, *Shewanella* spp.; Belchik et al 2011, Guha et al 2003), the search for Fe(III)-reducing extreme acidophiles may enable isolation of useful microorganism for metal bioremediation purposes. Another potential use of Fe(III)-reducing acidophiles in biohydrometallurgical application lies in their reductive dissolution of Fe(III)-minerals (e.g., nickel laterites where Ni is intimately associated with goethite (α -FeO·OH); Bridge and Johnson 1998, Bridge and Johnson 2000, Roden et al 2000, Hallberg et al 2011, Johnson 2012, Johnson et al 2013, Ñancucheo et al 2014). *At. ferrooxidans*, *Sb. benefaciens*, *Acidicaldus organivorans*, and *Acidiphilum* sp. SJH have been used to reduce soluble Fe(III), causing disequilibrium between Fe(III) in the mineral phase and the solution phase, thereby accelerating the mineral dissolution rate. *At. ferrooxidans* was shown to dissolve over 70% of Ni from the ore within 14 days (Hallberg et al 2011). The genus *Sulfolobus* is known to be a group of obligately aerobic, thermo-acidophilic sulfur-oxidizing archaea. They have been isolated from a number of sulfur-rich geothermal environments around the world (Brock et al 1972, Huber and Stetter 1991, Jan et al 1999, Zillig et al 1980). Either autotrophically or heterotrophically, utilization of sulfur is widely recognized in *Sulfolobus* spp. (*S. tokodaii*; Suzuki et al 2002, *S. metallicus*; Huber and Stetter 1991, *S. yangmingensis*; Jan et al 1999, *S. shibatae*; Grogan et al 1990 and *S. tengchongensis*; Xiang et al 2003). Some *Sulfolobus* spp. such as *S. tokodaii* and *S. metallicus* were reported to possess Fe(II) oxidation ability, and *S.*

metallicus was used as an iron/sulfur oxidizer in bioleaching application of chalcopyrite (Bathe and Norris 2007, Brock et al 1976, Gautier et al 2008, Vilcáez et al 2008, Zhu et al 2011). On the other hand, Fe(III) reduction ability in *Sulfolobus* is much less clear. This study aimed to reveal the microbial community structure of this geothermal hot spring and to enrich/isolate Fe(III)-reducing acidophiles potentially useful in biohydrometallurgical applications.

3.2 Materials and methods

3.2.1 Sample collection and analysis

Chinoike Jigoku is located in the hot spring town of Beppu on the southern island of Kyushu (33° 330' N; 131° 480'), and features its distinctively red-colored, highly acidic pond (Fig. 3.1). Samples of surface water and 70 cm-deep bottom sediment were collected at the identical location of the pond on July 1, 2013. In the sampling tube, red sediment occupied the upper layer 20 cm thick, below which was found a much denser gray sediment (70 cm). Parameters such as temperature, pH, Eh and dissolved oxygen (DO) were measured on-site for surface water sample. Elemental concentrations in surface water sample were determined by ICP-AES. Total organic carbon (TOC) content of sediment samples was analyzed after a hydrothermal pre-treatment in which teflon vessels containing a known amount of each sediment sample and 60% HNO₃ solution were placed in the microwave digestion system (Ethos Plus, Milestone) and heated to 230°C with 7°C/min increments, kept for 15 min at 230°C, and finally allowed to cool to room temperature. Sediment samples were freeze-dried overnight for X-ray fluorescence and X-ray diffraction analyses. To estimate copy numbers of the 16S rRNA gene in the samples, real-time PCR (TaqMan Mater; Light- Cyclor 1.5, Roche) was conducted on genomic DNAs extracted from each sample (Ultra Clean Water DNA isolation kit and Power Soil DNA isolation kit, MOBIO) using bacterial primers 341f (5'-TCTACGGAAGGCTGCAG-3') and 517r (5'-TTACCGCGGCTGCTGG-3') (94°C-30 s, 52°C-30 s, 72°C-30 s in 20 ml). *Escherichia coli* 16S rRNA genes (ranging from 2.7×10⁴ to 2.7×10⁷ copies) were used as standard.



Fig. 3.1 Photographic images of the Chinoike Jigoku (“Blood Pond Hell”) hot spring: Ground view (upper) and aerial view (lower). (Photos courtesy of Mr. Masafumi Kudo, Chinoike Jigoku).

3.2.2 Microbial community structure analysis

The presence of bacteria and archaea in the samples was investigated by conducting PCR on genomic DNA extracted from red sediment using bacterial (27f and 1492r; Okibe et al 2013) and archaeal (Arch21f (5'-TTCCGGTTGATCCYGCCGGA-3': Delong 1992) and 1492r) primers, respectively. After that, bacterial 16S rRNA gene fragments were PCR-amplified using 341f and 517r primers by Ex-taq DNA polymerase (Takara) to be ligated into pT7Blue T vector (Merck Millipore). They were then used for transformation, and positive *E. coli* colonies were screened by direct colony-PCR using universal primers; M13F-pUC (5'-GTTTTCCTCCAGTCACGAC-3') and M13R-pUC (5'-CAGGAAACAGCTATGAC-3'). The insert DNA sequences were analyzed by an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and compared with those from GenBank using the BLAST search tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

3.2.3 Enrichment cultivation, colony isolation and identification

Culture enrichment was conducted by inoculating an aliquot of red sediment in 300 mL Erlenmeyer flasks containing 200 mL HBS medium (pH 2.0 with H₂SO₄) containing 10 mM Fe(III) (added as Fe₂(SO₄)₃·nH₂O), 10 mM glucose and 0.01% (w/v) yeast extract (incubated without shaking at 65°C). After subculturing several times, colonies were isolated on solid medium (using the same medium composition, solidified with 8% gellan gum, and incubated at 70°C). The 16S rRNA genes from isolates were amplified by Touchdown-PCR (Okibe et al 2013) using 21f and 1492r primers. DNA sequencing was conducted by the Research Support Center, Graduate School of Medical Sciences, Kyushu University. The 16S rRNA gene sequences were analyzed by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The phylogenetic tree was constructed by the neighborjoining method with a boot-strap value of 1,000 using ClustalX v2.0 (Thompson et al 1997) and visualized by TreeView (Page 1996).

3.2.4 Characterization of *Sulfolobus* sp. GA1

3.2.4.1 Optimal pH and temperature for cell growth of *Sulfolobus* sp. GA1

Pre-grown cells of *Sulfolobus* sp. GA1 were inoculated into 100 mL Erlenmeyer flasks containing 50 mL of fresh HBS medium with 10 mM glucose and 0.01% yeast extract. To determine the initial optimal pH, initial media pH values were adjusted with H₂SO₄ to 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, and 4.0, and flasks were incubated aerobically (150 rpm, at 70°C). To determine the optimal temperature, flasks were incubated micro-aerobically (without shaking) at 45, 55, 60, 65, 70, 75, and 80°C (pH 2.0). Cell densities were monitored using a Thoma counting chamber, after which culture doubling times were determined.

3.2.4.2 Utilization of organic substrates of *Sulfolobus* sp. GA1

For organic substrates, pre-grown cells of *Sulfolobus* sp. GA1 were inoculated into 100 mL Erlenmeyer flasks containing 50 mL of fresh HBS medium (pH 2.0) containing trace elements (per L; 10 mg ZnSO₄·7H₂O, 1 mg CuSO₄·5H₂O, 1.09 mg MnSO₄·5H₂O, 1 mg CoSO₄·7H₂O, 0.39 mg Cr₂(SO₄)₃·7H₂O, 0.6 mg H₃BO₃, 0.5 mg Na₂MoO₄·2H₂O, 0.1 mg NaVO₃, 1 mg NiSO₄·6H₂O, 0.51 mg Na₂SeO₄, and 0.1 mg Na₂WO₄·2H₂O) and one of the following substrates (added at different concentrations to provide similar carbon equivalents):

- (i) Carbohydrates: sucrose (5 mM), glucose/galactose/fructose/mannose (10 mM), arabinose/xylose (12 mM), glyceraldehyde (20 mM);
- (ii) Alcohols: mannitol/sorbitol (10 mM), glycerol (20 mM);
- (iii) Other: tryptone soya broth/yeast extract (0.04%).

For elemental sulfur and tetrathionate, pre-grown cells were inoculated into 100 mL Erlenmeyer flasks containing 50 mL of HBS medium (pH 2.0) containing 0.01% yeast extract plus either sodium tetrathionate (Na₂S₄O₆·2H₂O at 0.1, 1 or 10 mM) or

elemental sulfur (S^0 at 0.04% (w/v)). The flasks were incubated micro-aerobically (without shaking, at 70°C). Cell densities were monitored using a Thoma counting chamber.

3.2.4.3 Fe(III) reduction test using *Sulfolobus* sp. GA1

Pre-grown cells of *Sulfolobus* sp. GA1 were inoculated into 50 mL serum bottles containing 50 mL of HBS medium (pH 2.0) with 10 mM glucose, 0.01% yeast extract and 10 mM Fe(III) (added as $Fe_2(SO_4)_3 \cdot nH_2O$). The serum bottles were incubated micro-aerobically (without shaking, at 70°C). Samples were regularly taken to monitor cell densities (Thoma counting chamber) and concentrations of total soluble Fe and Fe(II).

3.2.4.4 Fe(II) oxidation test using *Sulfolobus* sp. GA1 (growth-decoupled)

Cell suspensions (5.0×10^8 cells/mL) of *Sulfolobus* sp. GA1 were prepared by resuspending pre-grown cells in 200 mL of fresh HBS medium (pH 2.0) containing 10 mM Fe(II) (added as $Fe_2(SO_4)_3 \cdot nH_2O$), in 500 mL Erlenmeyer flasks. The flasks were incubated aerobically (120 rpm, at 70°C). Samples were regularly taken to monitor the concentrations of Fe(II) and total soluble Fe. Cell-free controls were run to distinguish between chemical Fe(II) oxidation and microbial Fe(II) oxidation.

3.2.4.5 Cr(VI) reduction test using cell suspension of *Sulfolobus* sp. GA1

Cell suspensions (1.0×10^9 cells/mL) of *Sulfolobus* sp. GA1 were prepared by resuspending pre-grown cells in 50 mL of fresh HBS medium (pH 2.5; in 50 mL Falcon tubes) containing 0.2 mM Cr(VI) and 1 mM glucose. The tubes were then incubated micro-aerobically (without shaking, at 65°C). Concentrations of total soluble Cr and Cr(VI) were regularly monitored.

3.3 Results and discussion

3.3.1 Sample analysis

The physico-chemical characteristics of surface water and sediment samples from Chinoike Jigoku hot spring are shown in Table 3.1 and Table 3.2, respectively. The red sediment sample taken from a 0-10 cm depth was soft colloidal material, whereas gray sediments were dense and hard. In fact, the red sediment had the highest moisture content and was enriched in Fe compared to gray sediments from the deeper zones (Table 3.2). TOC levels of sediment samples were over 10 times higher than that of the surface water, and had over 10-fold-greater 16S rRNA gene copy numbers (Tables 3.1 and 3.2). The soluble Fe concentration of surface water was unexpectedly low this time (0.9 mg/L), compared to numbers previously reported (2.2-5.17 mg/L in 1972-1998; Oue et al 2002). XRD analysis confirmed the presence of hematite (Fe_2O_3 ; red in color) and jarosite ($\text{KFe}_3(\text{OH})_6(\text{SO}_4)_2$; yellowish-red in color) in the red sediment (Fig. 3.3). Hematite peaks sequentially disappeared at deeper depth, corresponding to discoloration of the sediments from red to gray (Fig. 3.3b-d). The color of the Chinoike Jigoku hot spring was reported to be of a much darker red in the past, but has gradually become lighter (more yellowish than red) in recent years due to an increase in the content of jarosite in the sediments (Oue et al 2002).

Table 3.1 Characteristics of surface water sample from Chinoike Jigoku hot spring (as of July, 2013).

pH	Temp. (°C)	Eh (vs SHE) (mV)	DO (mg/L)	TOC (mg/mL)	16S rRNA gene copy (/mL)	Inorganic elements (mg/L)							
						Mg	Al	S	Ca	Mn	Fe	Zn	Cr/Cu/Pb
2.6	60	737	4.9	0.09	1.0×10^5	7.4	0.7	57.8	24	0.9	0.9	0.007	ND

"ND"; not detected. Detection limit values for Cr, Cu, and Pb are 2, 0.5, and 20 ng/mL, respectively.

Table 3.2 Characteristics of sediment samples from Chinoike Jigoku hot spring (as of July, 2013).

Depth (cm)	Red	Grey		
	sediment ^a	sediment ^a		
	0-10	20-30	40-50	60-70
TOC (mg/g)	1.3	1.2	2.8	1.0
Moisture content (%)	56	44	39	34
16S rRNA gene copy (/g)	2.2×10^6	4.3×10^6	3.8×10^6	2.1×10^6
Elemental composition (mass %)				
Na	1.6	ND	1.6	2.4
Mg	0.3	0.3	0.3	0.4
Al	16.1	16.9	15.2	15.7
Si	25.4	28.7	28.6	27.0
P	0.1	0.1	0.04	0.1
S	1.3	0.9	1.0	0.5
Cl	0.1	ND	0.8	ND
K	0.8	0.6	0.6	1.0
Ca	0.1	0.3	0.3	0.4
Ti	0.6	0.6	0.5	0.5
Mn	0.02	ND	ND	0.1
Fe	4.9	1.4	1.7	3.3
As	0.1	0.04	0.1	0.01

^a Sediments derived from the same origin as surface water (Table 1). "ND"; not detected. Detection limit values for Na, Cl, and Mn are 0.24, 0.018, and 0.006 mass %, respectively, in XRF measurement.

3.3.2 Microbial population analysis

The ferric mineral-rich red sediment sample was chosen for microbial community structure analysis, especially to search for Fe(III)-reducing microorganisms. Initial PCR amplification of 16S rRNA genes from environmental samples was successful with bacterial 27f/1492r primers, but unsuccessful with archaeal 21f/1492r primers. It was therefore suspected that bacteria were the main constituents of the microbial community, and random sequencing analysis was

Table 3.3 Microbial community profiling of Chinoike Jigoku hot spring (based on 177 bp-long 16S rRNA gene fragments amplified by bacterial primers).

Closest organism (based on 177-bp 16S rRNA gene fragment)	Ratio in clone library (%)	Species / Strain	Accession No.	Identity (%) (No. of clones)	No. of clones
<i>Propionibacterium</i> sp.	31.7	HUK23	AB453308	100(32), 99(3), 96(1), 95(1), 94(1), 81(1)	39
<i>Deftuviitoga</i> sp.	13.8	<i>D. tunisiensis</i> ^T	NR122085	100(9), 99(6), 98(1), 87(1)	17
<i>Clostridium</i> sp.	11.4	<i>C. elmenteitii</i> E2SE1-B	AJ271453	95(4), 92(1)	5
		<i>C. kluyveri</i> ^T	NR074447	94	1
		<i>C. pascui</i> ^T	NR026322	91	1
		<i>C. stercorarium</i> HL-3	KM036189	98	3
		F-02	AB504377	95	2
		IrT-R5M2-31	AJ291836	95(1), 94(1)	2
<i>Bacillus</i> sp.	8.1	<i>B. firmus</i> TY0125	KF477127	98	1
		<i>B. foraminis</i> FDS11	KM555278	97	1
		G5-6b clone 53.6	DQ357791	89(4), 88(3), 77(1)	8
<i>Anaerobaculum</i> sp.	7.3	<i>A. mobile</i> ^T	NR102954	100(5), 99(2), 98(1), 94(1)	9
<i>Labrys</i> sp.	7.3	<i>L. neptuniae</i> ^T	NR043801	100(6), 99(3)	9
<i>Thermoflavimicrobium</i> sp.	4.9	<i>T. dichotomicum</i> ^T	NR025005	93(4), 89(1), 88(1)	6
<i>Thermanaeromonas</i> sp.	4.1	IA 106	JQ446369	94(3), 91(1)	4
		<i>T. toyohensis</i> ^T	NR024777	91	1
<i>Alicyclobacillus</i> sp.	1.6	<i>Alb. acidoterrestris</i> ^T	NR118645	97	1
		<i>Alb. tolerans</i> PCG-3	KF940051	100	1
<i>Cellulosibacter</i> sp.	1.6	<i>C. alkalithermophilus</i> ^T	NR116826	98	2
<i>Saccharofermentans</i> sp.	1.6	<i>S. acetigenes</i> ^T	NR115340	94	2
<i>Acidithiobacillus</i> sp.	0.8	<i>At. ferrooxidans</i> ^T	NR114599	99	1
<i>Sulfobacillus</i> sp.	0.8	<i>Sb. acidophilus</i> ^T	CP003179	100	1
<i>Dehalobacter</i> sp.	0.8	<i>D. restrictus</i> ^T	NR121722	95	1
<i>Desulfotomaculum</i> sp.	0.8	<i>D. thermocisternum</i> ^T	NR117746	92	1
<i>Desulfuribacillus</i> sp.	0.8	<i>D. alkaliarsenatis</i> ^T	NR126221	89	1
<i>Sedimentibacter</i> sp.	0.8	<i>S. hongkongensis</i> KI	AY571338	87	1
<i>Singulisphaera</i> sp.	0.8	<i>S. mucilaginoso</i> Z-0071	HM748856	99	1
<i>Methanothermobacter</i> sp.	0.8	<i>M. marburgensis</i> ^T	NR102881	97	1
Total number of genera: 19				Total number of clones: 123	

performed using bacterial-specific 341f/517r primers. Results obtained from approximately 177 bp-long 16S rRNA gene sequences from 123 clones are shown in Table 3.3: A total of 19 prokaryotes, including one archaeon (based on the 177-bp 16S rRNA gene fragment), were shown to be the closest relatives to organisms representing the microbial diversity of the hot spring. Most of them were related to known neutrophilic (some even alkaliphilic) anaerobes, probably originating from neutral Na-Cl thermal water before mixing with acidic H-SO₄ thermal water to form the hydrothermal system beneath the Chinoike Jigoku hot spring (Oue et al 2002). Known Fe metabolizing extreme acidophiles (*Acidithiobacillus* sp., *Sulfobacillus* sp. and *Alicyclobacillus* sp.; Lu et al 2010) represented only 3.2% of the clone library. Although the spring water features high temperature of 60°C, mesophilic species such as

Acidithiobacillus sp. were detected as the closest organisms. These mesophiles likely thrived alongside the formation of H-SO₄ thermal water from infiltrated meteoric waters (Oue et al 2002). However, given the relatively low 16S rRNA gene sequence identities obtained from a number of clones (partly due to the short DNA sequence analyzed in this study), their actual characteristics are unclear.

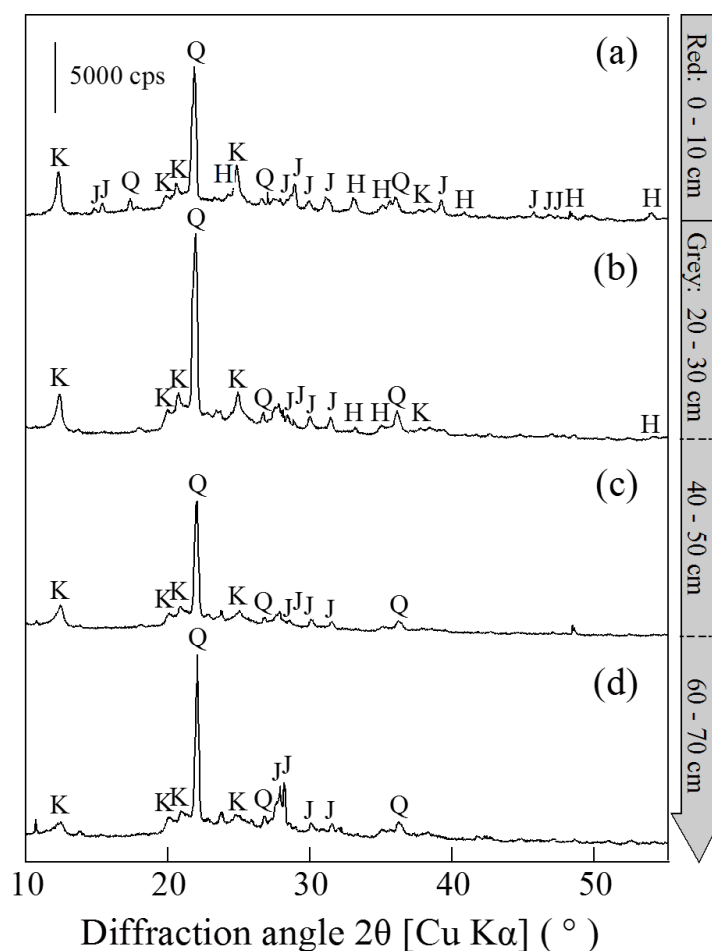


Fig. 3.2 X-ray diffraction patterns of Chinoike Jigoku sediment samples taken from different depths: red sediment from 0 to 10 cm depth (a) and gray sediments from 20 to 30 cm (b), 40 to 50 cm (c) and 60 to 70 cm (d) depths. H; hematite (ICSD 41541), J; jarosite (ICSD 166801), Q; quartz (ICSD 170554), K; kaolinite (ICSD 63315).

3.3.3 Phylogenetic analysis for *Sulfolobus* sp. GA1

Culture enrichment with Fe(III) under micro-aerobic conditions was repeated several times by visibly confirming discoloration of the orange Fe(III) ion each time. Several single white colonies were eventually obtained on Fe(III)-containing solid medium, each of which was then transferred into liquid medium to screen for its Fe(III)-reducing ability. Among them, isolate GA1 displayed the most effective Fe(III)-reduction in the preliminary test. Sequence of 16S rRNA (1381 base pair) of isolate GA1 was analyzed and shown below.

5'-

AGCCATGGGAGTCGTACGCTCCCGGGCAAGGGAGCGTGGCGGACGGCTG
AGTAACACGTGGCTAACCTACCCTGAGGAGGGAGATAACCCCGGGAACT
GGGGATAATCTCCCATAGGCGAGGAGTCCTGGAACGGTTCCTCGCTGAAA
GGTTCATGGGCTATTTCCCGCTCATGAGCGCCTCAGGATGGGGCTGCGGCC
CATCAGGTAGTTGGGGGGGTAAGGGCCCCCAAGCCTATAACGGGTAGGG
GCCGTGAGAGCGGGAGCCCCAGTTGGGCACTGAGACAAGGGCCCAGGC
CCTACGGGGCGCACCAAGGCGCGAAACGTCCCCAATGCGCGGAAGCGTGA
GGGCGTCACCCCGAGTGCTCCCGTAAGGGAGCTTTTCCCGCTCTACAaAg
GcGGGGGAATAAGCGGGGGGgCAAGTCTGGTGTGTCAGCCGCCGCGGTAATACC
AGCCCCGCGAGTGGTTCGGGACTCTTACTGGGCcTAAGGCGCCCGTAGCCG
GCCCCACAAGTCACTCCTTAAAGACCTCGGCTCAACCGGGGGGAATGGGGG
TGAtACTGTCTGGGCTAGGGGGCGGGAGAGGCCAGCGGTACTCCCGGAGTA
GGGGCGAAATCCTCAGATCTCGGGAGGACCACCAGTGGCgAAAGCGGCTG
GCTAgAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGCAGCAAAAGGG
ATTAGATACCCCTGTAGTCCCGGCTGTAAACGATGCAGGCTAGGTGTCACA
TGGGCTTAGAGCCCATGTGGTGCCGCAGGGAAGCCGTTAaGCCTGCCGCCT
GGGGAGTACGGTCGCAAGACTGAACTTAAAGGaaTTggcGGGGGAGCACC
ACAAGGGGGTGGAAACCTGCGGCTCAATTGGAGTCAACGCCTGGAATCTTAC
TAGGGGAGACCGCAGGATGACGGCCAGGCTAACGACCTTGCCTGACTCGC
GGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGTTGTGAAATGTCCTGTT
AAGTCAGGCAACGAGCGAGACCCCCACCACTAGTTGGTATCCTGGTCTCC
GGGCCGGGACCACACTAGTGGGACTGCCGGCGTAAGCCGGAGGAAGGAG

GGGGCCACGGCAGGTCAGCATGCCCCGAAACCCCTGGGCCGCACGCGGG
TTACAATGGCAGGGACAGCGGGATTCCGACCCCGAAAGGGGAAGGTAATC
CCTTAAACCCTGCCGCAGTTGGGATCGAGGGCTGAAACTCGCCCTCGTGA
ACGAGGAATCCCTAGTAACCGCAGATCAACAATCTGCGGTGAATACGTCCC
TGCTCCTTGCACACACCGCCCGTCGCTCCACCCGAGTAGGAGAGGGGTGA
GGCCCCTTGCCTTTAGGTGGGGGGTCGAGC -3'

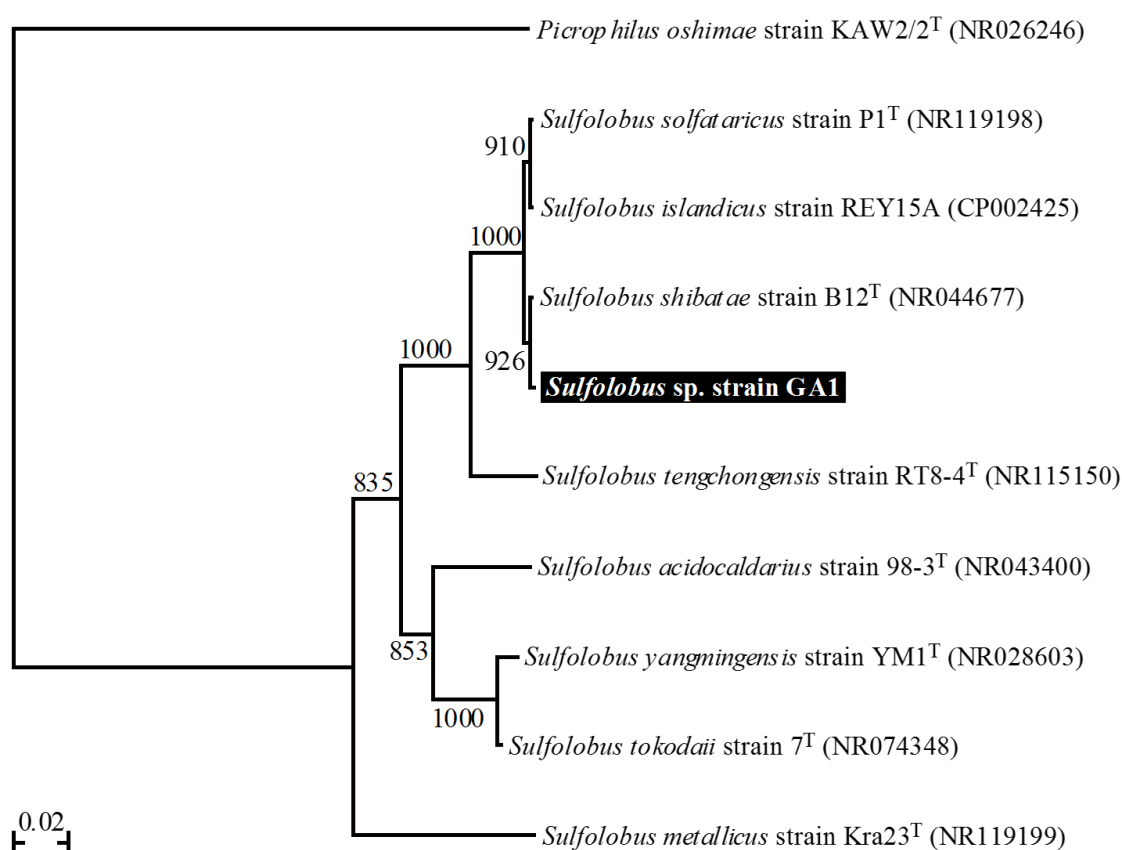


Fig. 3.3 Phylogenetic tree of strain GA1 in relation to known *Sulfolobus* spp. based on 16S rRNA gene sequences, constructed by the neighbor joining method (*Picropilus oshimae* KAW2/2T as outgroup). Numbers at nodes indicate bootstrap values for 1,000 replicates of the original dataset. Values in brackets indicate NCBI accession numbers and 16S rRNA gene sequence identities with strain GA1. Scale bar indicates number of nucleotide substitutions per site.

16S rRNA gene sequence analysis of isolate GA1 (1465 bp) confirmed it to be closely related (99.7% gene identity) to *S. shibatae* B12^T (Fig. 3.3). Since initial PCR failed to amplify archaeal 16S rRNA genes from environmental samples, this result was somewhat unexpected. Nonetheless, overall results revealed that wide microbial diversity (including both bacterial and archaeal domains) exists in the Chinoike Jigoku hot spring. The fact that the amount of soluble Fe in surface water was highly limited (Table 3.1) indicates that some Fe-metabolizing microorganisms at the site may rely on ferric minerals such as hematite and jarosite (Fig. 3.2) as electron acceptors.

3.3.4 Characterization of *Sulfolobus* sp. GA1

Strain GA1 was found to grow in a pH range of 1.25-4.0, with optimal initial pH of 2.9 ($T_d = 4.4$ h; Fig. 3.4a). No cell growth was observed at pH 1.0. The temperature range for strain GA1 growth was between 55 and 80°C, with an optimal temperature of 70°C ($T_d = 5.6$ h; Fig. 3.4b). No growth was observed at 45°C. Strain GA1 was unable to grow autotrophically on Fe(II), S^0 or tetrathionate. Likewise, neither S^0 nor tetrathionate promoted heterotrophic growth of strain GA1 on 0.01% yeast extract, and no pH decrease (as a result of sulfur oxidation) was evident. Rather, strain GA1 growth was weaker when sulfur compounds were provided in addition to yeast extract (Fig. 3.5). *Sulfolobus* spp. often occurs in acidic geothermal springs, water holes or mud ponds containing elemental sulfur (Brock et al 1972, Huber and Stetter 1991, Jan et al 1999, Zillig et al 1980). Sulfur oxidation (either autotrophically or heterotrophically) is considered a feature of *Sulfolobus* sp. (yet with some ambiguity for *S. acidocaldarius* and *S. solfataricus*; Table 3.4). However, strain GA1 might have lost or did not attain this trait during evolution in the environment in which sulfur exists solely in the most oxidized form, SO_4^{2-} (Maeno 2015). Strain GA1 was found to grow heterotrophically on several carbohydrates (especially readily on glucose) as sole energy source. The best growth was observed in complex medium with yeast extract or tryptone soya broth (Table 3.4).

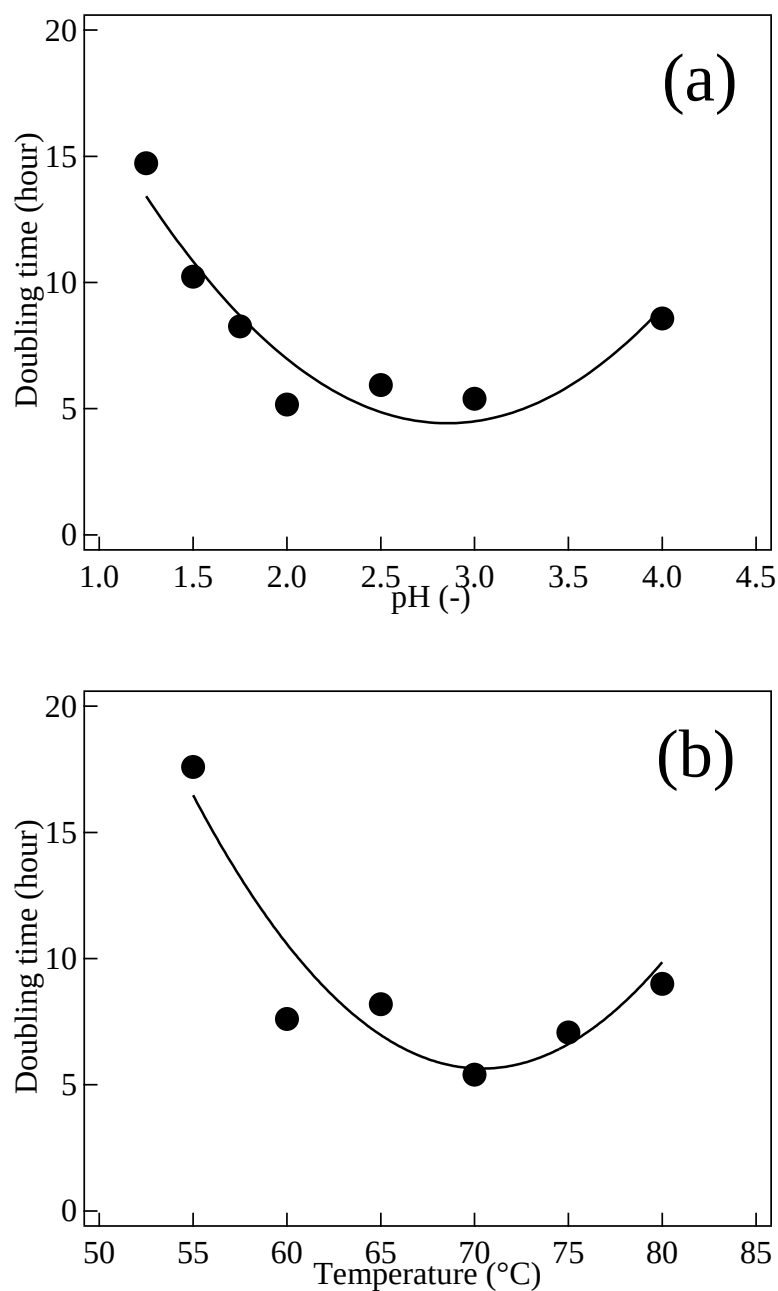


Fig. 3.4 Effect of pH (a) and temperature (b) on growth of strain GA1. Each culture doubling time is the mean of duplicate experiments.

Table 3.4 Characteristics of strain GA1 and known *Sulfolobus* sp. type strains.

DSM No. Accession No.	Strain GA1	<i>S. shibatae</i> B12 ^T DSM 5389 NR044677	<i>S. tokodaii</i> 7 ^T DSM 16993 NR074348	<i>S. acidocaldarius</i> 98-3 ^T DSM 639 NR043400	<i>S. solfataricus</i> P1 ^T DSM1616 NR119198	<i>S. metallicus</i> Kra23 ^T DSM 6482 NR119199	<i>S. yangmingensis</i> YM1 ^T NR028603	<i>S. tengchongensis</i> RT8-4 ^T NR115150
<i>pH range (optimal)</i>	1.25-4.0 (2.9)	2.5-5.0 (3.0-4.0) ^a	2.0-5.0 (2.5-3.0)	1.0-5.9 (2.0-3.0) ^c	2.0-5.0 (4.5) ^{a,d}	1.0-4.5	2.0-6.0 (4.0)	1.7-6.5 (3.5)
<i>Temperature range (optimal; °C)</i>	55-80 (70)	65-86 (81) ^{a,b}	70-85 (80)	55-85 (70-79) ^{a,e}	55-85 (70-79) ^{a,e}	50-75 (65)	65-95 (80)	65-95 (85)
<i>Substrates</i>								
Sulfur compounds								
Elemental sulfur	—	+	+	± ^{b, e, f}	± ^{b, e, f}	+	+	+
Tetrathionate	—	•	•	+ ^e	+ ^e	•	+	•
Carbo- hydrates	Sucrose	+	•	+ ^a	+ ^a	—	+	+
	Glucose	+	+	+ ^a	+ ^a	—	+	—
	Galactose	+	•	— ^a	+ ^e	—	+	+
	Fructose	—	•	+ ^e	+ ^a	—	+	+
	Mannose	+	•	+ ^e	+ ^a	—	+	—
	Arabinose	+	•	+ ^e	+ ^a	—	+	+
	Xylose	—	•	— ^a	+ ^a	—	+	+
	Glyceraldehyde	+	•	•	•	•	•	•
Alcohols	Mannitol	—	— ^a	— ^a	— ^a	•	•	•
	Sorbitol	—	— ^a	— ^a	— ^a	•	+	—
	Glycerol	—	— ^a	— ^a	— ^a	•	•	•
Others	Yeast extract	+	+ ^b	+ ^e	+ ^e	—	+	+
	Tryptone	+	+ ^b	+	+ ^a	—	•	+
<i>Reference</i>	This study	[a, b]	[g]	[a, b, c, e, f]	[a, b, d, e, f]	[h]	[i]	[i]

"±"; growth observed, "•"; no growth observed (in case of strain GA1, no increase in cell number after 1 month incubation), "—"; no data.

^a b c d e g h i, and ^j; data retrieved from Grogan 1989, Grogan et al 1990, Brock et al 1972, Zillig et al 1980, Takayanagi et al 1996, Suzuki et al 2002, Huber and Stetter 1991, Jan et al 1999, and Xiang et al 2003, respectively. ^f; S oxidation ability was reported to have lost as a result of possible mutation during subculturing [Huber et al 2006]. Expression of S oxidation may therefore rely on sub-culturing conditions.

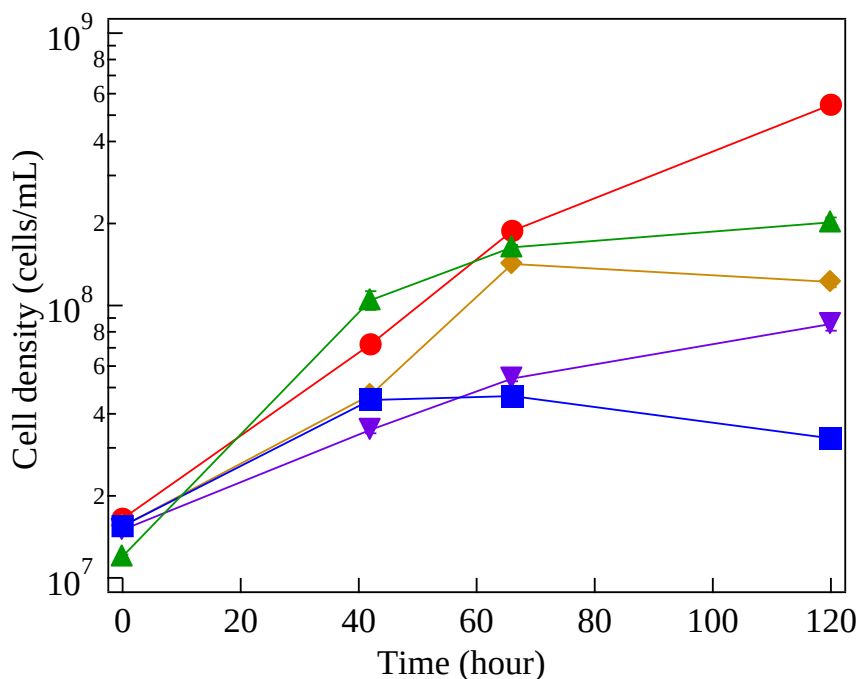


Fig. 3.5 Cell growth of strain GA1 on 0.01% yeast extract (y.e.) with or without sulfur compounds: y.e. only (●); y.e. plus 0.02% elemental sulfur (▲); y.e. plus 0.1 mM tetrathionate (◆), y.e. plus 1 mM tetrathionate (▼); y.e. plus 10 mM tetrathionate (■). Data points are mean values from duplicate experiments. Errors bars depicting averages are not visible, as they were smaller than the data point symbols in all cases.

3.3.5 Fe metabolism of *Sulfolobus* sp. GA1

Fe(II) oxidation ability has been reported for a few *Sulfolobus* spp. *S. tokodaii* was found to readily oxidize Fe(II) during heterotrophic growth on yeast extract (90% of 50 mM Fe(II)), but its Fe(II) oxidation during autotrophic growth was much weaker than that by *S. metallicus* (Bathe and Norris 2007). Although Brock et al. (1976) observed Fe(II) oxidation in waters from Yellowstone hot springs that included significant populations of *S. acidocaldarius*, whether or not this observation indeed derived from microbial Fe(II) oxidation by this specific species is unclear. In the case of strain GA1, Fe(II) oxidation was observed, but only to a minor extent. In growth-decoupled cell suspensions, 1.3 mM Fe(II) was oxidized in 6 days, while 0.3 mM Fe(II) was abiotically oxidized (Fig. 3.6).

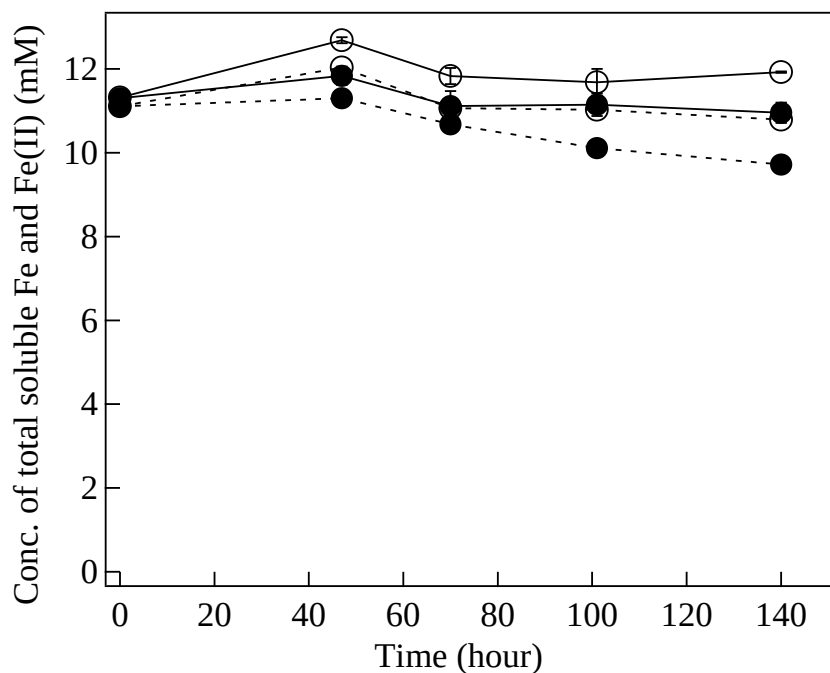


Fig. 3.6 Changes in concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) (●; strain GA1 cell suspension of 5.0×10^8 cells/mL, ○; sterile controls). Data points are mean values from duplicate experiments. Error bars depicting averages are not visible as they were smaller than the data point symbols in all cases.

Strain GA1 readily reduced Fe(III) to Fe(II) during growth on glucose under micro-aerobic conditions (Fig. 3.7). Only limited information is available thus far on Fe(III) reduction ability in *Sulfolobus* spp. Brock and Gustafson reported that a *Sulfolobus* sp. reduced soluble Fe(III) to Fe(II) under aerobic conditions in the presence of glutamate or elemental sulfur as the electron donor (Brock and Gustafson 1976); the authors quoted it to be *S. acidocaldarius* in that paper, although this is unlikely, since this archaeon does not grow autotrophically, as indicated by Johnson et al. (Johnson et al 2012).

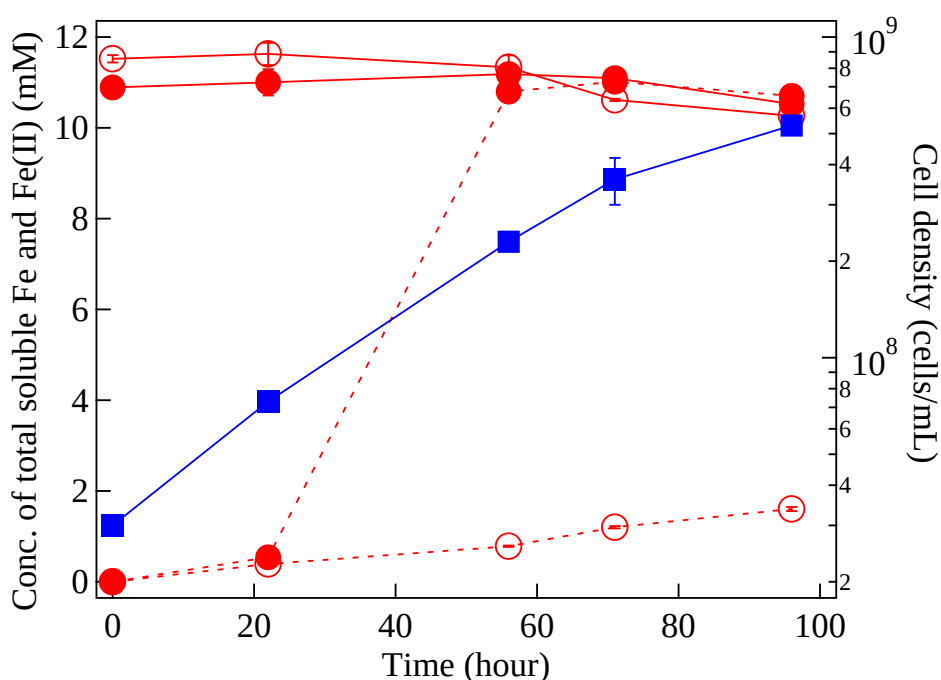


Fig. 3.7 Changes in concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) (●; inoculated, ○; sterile; left axis) during micro-aerobic growth of strain GA1 (■; right axis). Data points are mean values from duplicate experiments. Error bars depicting averages are not visible as they were smaller than the data point symbols in all cases.

3.3.6 Cr(VI) reduction of *Sulfolobus* sp. GA1

Strain GA1 was tested for its ability to catalyze reduction of Cr(VI) (as chromate; CrO_4^{2-}), which is both soluble and highly toxic (Tandon et al 1984). The concentration of Cr(VI) readily decreased (especially with glucose as electron donor) in cell suspension of strain GA1 (Fig. 3.8). The fact that no decrease in the total soluble Cr concentration was observed in cell suspension indicates that Cr(VI) was microbially reduced to less toxic/soluble Cr(III), and this was also qualitatively confirmed by X-ray absorption near-edge structure spectra (data shown in Chapter 4). This is the first report showing Cr(VI) reduction capability in *Sulfolobus* spp.

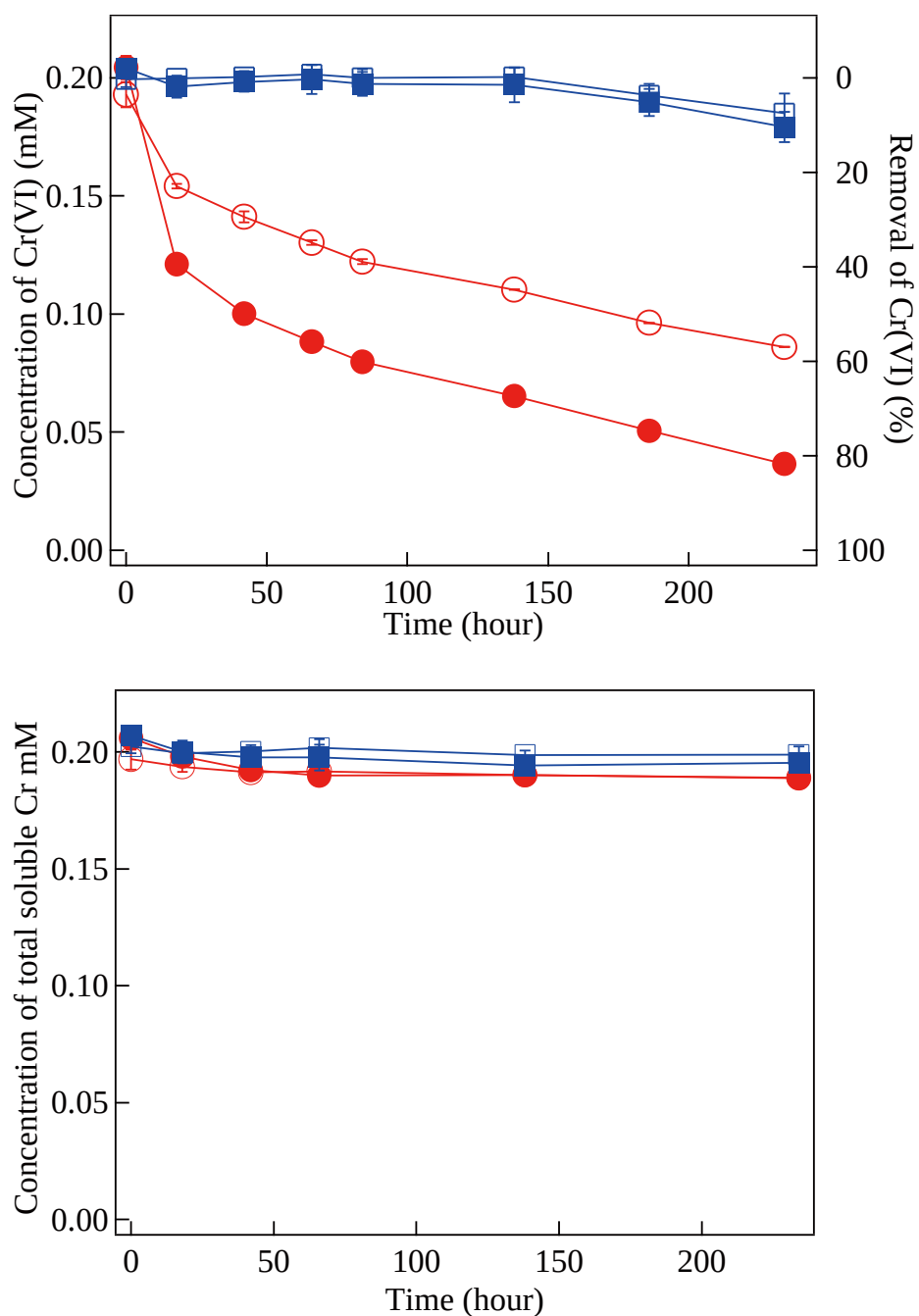


Fig. 3.8 Cr(VI) reduction (upper) and total soluble Cr removal (bottom) in cell suspensions of strain GA1 (●, ○) and in cell free controls (■, □), with (solid symbols) or without (open symbols) glucose as electron donor.

3.4 Conclusions

Wide microbial diversity was evident at the Chinoike Jigoku hot spring and included both bacteria and archaea. Successive culture enrichment with Fe(III) under micro-aerobic conditions resulted in isolation of *Sulfolobus* sp. GA1. Unlike most *Sulfolobus* spp., strain GA1 is incapable of sulfur oxidation and grows heterotrophically on glucose and other organic substrates. Its Fe(II) oxidation ability was only minor. Strain GA1 was shown to readily reduce Fe(III) during growth on glucose under micro-aerobic conditions, and was also found to reduce highly toxic Cr(VI) to less toxic/soluble Cr(III), demonstrating its potential utility in metal bioremediation.

References

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Data shown in this chapter are partially included in the paper submitted to Research in Microbiology (2016, 167, 595-603) entitled “Microbial community profiling of the Chinoike Jigoku (“Blood Pond Hell”) hot spring in Beppu, Japan: Isolation and characterization of Fe(III)-reducing *Sulfolobus* sp. strain GA1”.

Chapter 4

Iron redox transformation by the thermo-acidophilic archaea, *Sulfolobus* species

Abstract

Iron redox transformations by six *Sulfolobus* strains (*S. metallicus* Kra23; *S. tokodaii* 7; *S. acidocaldarius* 98-3; *S. solfataricus* P1; *S. shibatae* B12; *Sulfolobus* sp. GA1) were studied to clarify the general trend of Fe metabolism across different species of the genus *Sulfolobus*. Fe(II) oxidation was detected in cell-suspensions of all six strains though to a highly varying extent, with *S. metallicus* Kra23 and *S. tokodaii* 7 being the most effective, *S. acidocaldarius* 98-3 being moderate, and *S. solfataricus* P1, *S. shibatae* B12 and *Sulfolobus* sp. GA1 being marginal to minor Fe(II)-oxidizers. Whilst Fe(III) reduction was observed in all strains, Fe(III)-reducing ability was differently expressed in each strain with strong dependence on the oxygen level and growth status: Growth-uncoupled cell-suspensions of all strains, except for *Sulfolobus* sp. GA1, readily reduced Fe(III) either/both anaerobically or/and micro-aerobically. Four strains (*S. tokodaii* 7, *S. solfataricus* P1, *S. shibatae* B12 and *Sulfolobus* sp. GA1) were capable of anaerobic growth using Fe(III) as the sole electron acceptor. Fe(III) reduction by *Sulfolobus* sp. GA1 was thus found solely growth-dependent. In addition to Fe redox reactions their reducing abilities of highly toxic hexavalent chromium were tested. Cell-suspensions of all strains were capable of reducing Cr(VI) to Cr(III) under micro-aerobic conditions. The trend in degree of Fe(III) reduction by six strains did not necessarily corresponded to that of Cr(VI) reduction.

4.1 Introduction

The genus *Sulfolobus* (*S.*) is generally recognized as a group of aerobic, thermo-acidophilic sulfur-oxidizing archaea. They naturally occur in a number of sulfur-rich acidic geothermal environments (Brock et al 1972, Huber and Stetter 1991, Jan et al 1999, Zillig et al 1993). Consequently, utilization of S^0 (either autotrophically or heterotrophically) is widely seen in known *Sulfolobus* spp. (*S. tokodaii*; Suzuki et al 2002, *S. metallicus*; Huber and Stetter 1991, *S. yangmingensis*; Jan et al 1999, *S. shibatae*; Grogan et al 1990, *S. tengchongensis*; Xiang et al 2003), although with an exception of non-sulfur oxidizing *Sulfolobus* sp. GA1, as was recently reported (Masaki et al 2016).

Given that microbial Fe(II) oxidation plays a major role in the geochemical Fe cycle of highly acidic environments, and that a number of *Sulfolobus* species are widely distributed in such environments, there is yet only limited study reporting Fe(II) oxidation ability in this genus (*S. metallicus*, *S. tokodaii*; Bathe and Norris 2007). Owing to its both Fe(II)- and S^0 -oxidizing metabolisms, *S. metallicus* has been employed to apply for high-temperature bioleaching (oxidative dissolution) of sulfide minerals such as pyrite and chalcopyrite (Sandström et al 2005, Gautier et al 2008, Vilcáez et al 2008, Zhu et al 2011). A comprehensive search for Fe(II) oxidation ability among different *Sulfolobus* species would therefore lead to better understandings of the role of this genus in natural Fe cycles, as well as in possible utilities of the genus for application purposes.

So far, Fe(III)-reducing ability in the genus *Sulfolobus* is even less clarified than its Fe(II)-oxidizing ability: *S. acidocaldarius* was reported to reduce soluble Fe(III) to Fe(II) under aerobic conditions in the presence of elemental sulfur or glutamate as the electron donor (Brock and Gustafson 1976), although it was controversially pointed out that this archaeon does not grow autotrophically (Johnson et al 2012). Our previous study detected the Fe(III)-reducing ability during growth under micro-aerobic conditions in recently isolated *Sulfolobus* sp. GA1 (Masaki et al 2016). This observation thus further motivated us to investigate general trend of Fe(III)-reducing ability across different species of the genus *Sulfolobus*.

The possible importance of sulfur- and iron-oxidizing acidophiles in the natural geochemical Fe cycle by means of not only Fe(II) oxidation but also Fe(III) reduction

was first pointed out by Brock and Gustafson (1976), by showing Fe(III)-reducing ability in *At. thiooxians*, *At. ferrooxidans* and *S. acidocaldarius*. Later studies in fact revealed a variety of acidophiles involved in microbial Fe(III) reduction: Anaerobic growth via Fe(III) respiration using S^0 (or other reduced inorganic sulfur compounds) as electron donor was reported for *At. ferrooxidans* (Ohmura et al 2002), *At. ferrivorans* (Hallberg et al 2010), and *Acidiferrobacter thiooxydans* (Hallberg et al 2011b). The ability to use Fe(III) as a sole electron acceptor during heterotrophic growth was reported in *Sulfobacillus* (*Sb.*) *acidophilus*, *Sb. thermosulfidooxidans*, *Acidimicrobium ferrooxidans* (Bridge and Johnson 1998) and *Acidicaldus organivorus* (Johnson et al 2006). Reductive dissolution of Fe(III) minerals was observed in micro-aerobic cultures of heterotrophs, *Acidiphilium* spp. (Johnson and Mcginness 1991, Johnson and Bridge 2002, Coupland and Johnson 2008), *Acidocella* spp. and *Acidobacterium* spp. (Coupland and Johnson 2008).

Based on the above biodiversity found so far, it would be reasonable to expect that Fe(III)-reducing abilities can also exist across different *Sulfolobus* spp., especially given the fact that acido-thermophilic *Sulfolobus* spp. naturally inhabit in environments of both acidic pHs (where Fe(III) becomes highly bioavailable) and high-temperatures (where solubility of oxygen becomes lower; e.g., oxygen solubility in water at 70°C is approximately one-half of that at 25°C; Lange 1956).

From the viewpoint of biohydrometallurgical application, search for microbial Fe(III)-reducing abilities would benefit the development of bio-processes such as bioremediation of toxic metals (e.g., hexavalent chromium; Masaki et al 2015, Cummings et al 2007) and reductive bioleaching of Fe(III)-minerals (e.g., nickel laterites where Ni is associated with goethite; Hallberg et al 2011a, Johnson 2012, Johnson et al 2013, Ñancucheo et al 2014).

Overall, with the aim to clarify their roles in natural geochemical Fe cycles as well as their potentials in biohydrometallurgical applications, we have chosen representative *Sulfolobus* spp., namely *S. metallicus* Kra23, *S. tokodaii* 7, *S. acidocaldarius* 98-3, *S. solfataricus* P1, *S. shibatae* B12, as well as *Sulfolobus* sp. GA1, to test and compare their Fe transformation (Fe(II)-oxidation and Fe(III)-reduction) capabilities. Their capabilities in Cr(VI) reduction were also investigated to compare with those in Fe(III) reduction.

4.2 Materials and methods

4.2.1 Fe(II) oxidation test using growth-uncoupled cell-suspensions under aerobic conditions

Each of the 6 strains was pre-grown as described in the previous section (500 mL Erlenmeyer flasks containing 200 mL medium), harvested at the late exponential phase by centrifugation (12,000 g, 10 min), washed twice, and finally re-suspended in 200 mL of fresh HBS medium (in 500 mL Erlenmeyer flasks; pH 2.0) containing 10 mM Fe(II) (as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$): Densities of the resultant cell-suspensions were approx. 5×10^8 cells/mL for *S. tokodaii* 7, *S. shibatae* B12 and *Sulfolobus* sp. GA1, and 2×10^8 cells/mL for *S. metallicus* Kra23, *S. acidocaldarius* 98-3 and *S. solfataricus* P1. Cell-suspensions were incubated shaken (70°C, 120 rpm) and samples were regularly taken to monitor the concentrations of Fe(II) and total soluble Fe.

The Fe(II) oxidation rates (mg-Fe/h; for comparison between abiotic and biotic Fe(II) oxidations) and specific Fe(II) oxidation rates (mg-Fe/h/cell; for comparison between *Sulfolobus* strains) were calculated.

4.2.2 Fe(III) reduction test using growth-uncoupled cell-suspensions under anaerobic / micro-aerobic conditions

Anaerobic experiments: Each of the 6 strains was pre-grown, harvested, washed (as described in the previous section), and finally re-suspended (to a final cell density of 1×10^9 cells/mL) in 10 mL of N_2 -purged HBS medium (in 25 mL serum bottles with rubber stoppers; pH 2.0) containing 10 mM Fe(III) (as $\text{Fe}_2(\text{SO}_4)_3 \cdot n\text{H}_2\text{O}$). Either 1 mM glucose (N_2 -purged) or 0.01% (w/v) elemental sulfur (for *S. metallicus* Kra23) was added as electron donor.

Micro-aerobic experiments: The preparation procedures followed the above anaerobic experiments except that 15 mL cell-suspensions were prepared in 15 mL Falcon tubes. None of the reagents were N_2 -purged.

All cell-suspensions (both anaerobic and micro-aerobic) were incubated at 70°C without shaking. Samples were regularly taken (using syringe needles for anaerobic experiments) to monitor concentrations of Fe(II) and total soluble Fe. All of the

experiments were conducted in duplicates.

The specific Fe(III) reduction rates (mg-Fe/h/ 1×10^{10} cells for anaerobic experiments; mg-Fe/h/ 1.5×10^{10} cells for micro-aerobic experiments) were calculated.

4.2.3 Growth-coupled dissimilatory Fe(III) reduction test under anaerobic conditions

All solutions were purged with N₂ until the DO (dissolved oxygen) level reached < 0.1 mg/L. Each of the 6 strains was pre-grown and inoculated (to a final cell density of 1×10^7 cells/mL) into 20 mL of HBS medium (in 25 mL serum bottles with rubber stoppers; pH 2.0) containing either 0.01% (w/v) S⁰ and trace elements (Masaki et al 2016) (for *S. metallicus* Kra23 only), or 10 mM glucose and 0.01% (w/v) yeast extract (for other 5 strains). The headspace of serum bottles was flushed with N₂ (80%)-CO₂ (20%) gas for obligately autotrophic *S. metallicus* Kra23 cultures. Different initial concentrations of Fe(III) (0, 5, 10, or 15 mM) were added to the growth media, which were then incubated shaken (70°C, 120 rpm). Samples were regularly taken using syringe needles to monitor cell densities (Thoma counting chamber) and concentrations of Fe(II) and total soluble Fe. All of the experiments were carried out in duplicates.

4.2.4 Cr(VI) reduction test using growth-uncoupled cell-suspensions under micro-aerobic conditions

Cell-suspensions (1×10^9 cells/mL; in 50 mL HBS medium in 50 mL Falcon tubes) were prepared for each strain as described in previous sections. One millimolar glucose (0.01% (w/v) S⁰, instead of glucose, for *S. metallicus* Kra23) and 0.2 mM Cr(VI) (as Na₂CrO₄·4H₂O) were added to the cell-suspensions, which were then incubated at 70°C without shaking. Samples were regularly taken to monitor concentrations of Cr(VI) and total soluble Cr. All of the experiments were carried out in duplicates.

4.2.5 Cr(VI) reduction and Cr immobilization using high density cell-suspensions of *Sulfolobus* sp. strain GA1 and *S. shibatae* B12 under micro-aerobic conditions

Cells of *Sulfolobus* sp. GA1 and *S. shibatae* B12 were pre-grown, harvested, washed, and then re-suspended to make a final density of 1×10^{10} cells/mL in 50 mL of HBS medium (pH 2.5) containing 0.2 mM Cr(VI) (or 0.2 mM Cr(III) as a control) and 1 mM glucose. Falcon tubes (50 mL) containing the above 50 mL reaction medium were maintained at 70°C without shaking. Samples were regularly taken after compensation of water evaporation with deionized water, to monitor concentrations of Cr(VI) and total soluble Cr. All of the experiments were carried out in duplicate. Cell pellets collected by filtration at the end of the experiment were applied to X-ray absorption near edge structure (XANES) analysis.

4.3 Results and discussion

4.3.1 Comparison of Fe(II) oxidation by six *Sulfolobus* strains in growth-uncoupled cell-suspensions under aerobic conditions

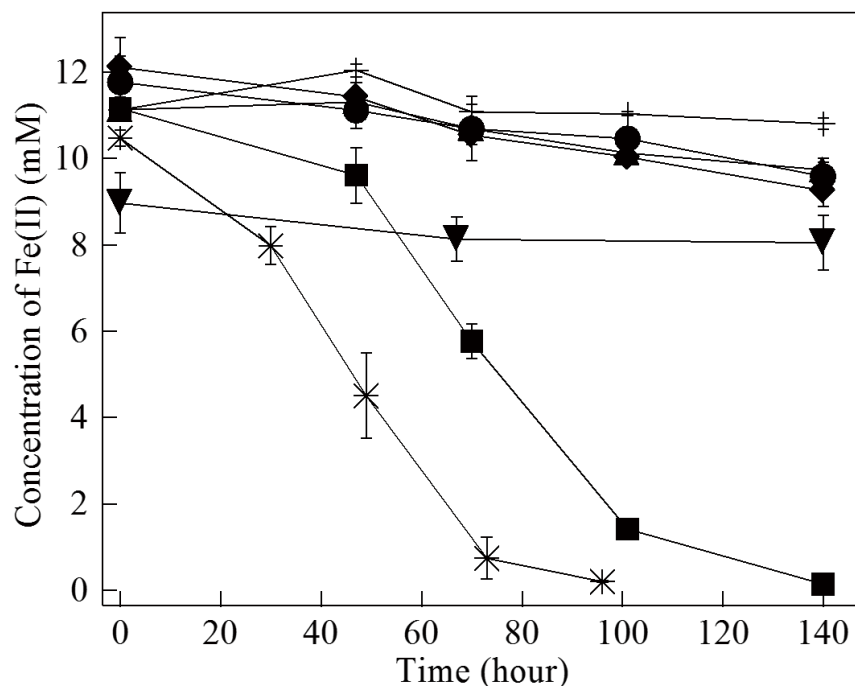


Fig. 4.1 Growth-uncoupled Fe(II) oxidation in cell-suspensions of six *Sulfolobus* strains (pH 2.0, 70°C, aerobic conditions): *S. metallicus* Kra23 (*); *S. tokodaii* 7 (■); *S. acidocaldarius* 98-3 (◆); *S. solfataricus* P1 (▼); *S. shibatae* B12 (●); *Sulfolobus* sp. GA1 (▲); sterile controls (+). The initial cell densities of cell-suspensions (naturally achieved after pre-growing each strain) were 2×10^8 cells/mL (*S. metallicus* Kra23; *S. acidocaldarius* 98-3; *S. solfataricus* P1) and 5×10^8 cells/mL (*S. tokodaii* 7; *S. shibatae* B12; *Sulfolobus* sp. GA1). Data points are mean values from duplicate experiments. Error bars depicting averages are not visible in some cases as these were smaller than the data point symbols.

Table 4.1 Fe(II) oxidation rates and specific Fe(II) oxidation rates determined for six *Sulfolobus* strains in growth-uncoupled cell suspensions under aerobic condition.

<i>Sulfolobus</i> strains	<i>S. metallicus</i> Kra23	<i>S. tokodaii</i> 7	<i>S. acidocaldarius</i> 98-3	<i>S. solfataricus</i> P1	<i>S. shibatae</i> B12	<i>Sulfolobus</i> sp. GA1	Cell-free controls
Fe(II) oxidation rates* ($\times 10^{-2}$ mg-Fe/h)	149 ± 10^a	86 ± 6.9^b	25 ± 7.7^b	14 ± 3.2^c	12 ± 7.1^b	6.9 ± 0.27^b	1.8 ± 0.85^a
Specific Fe(II) oxidation rates* ($\times 10^{-12}$ mg-Fe/h/cell)	37 ± 2.5^a	8.6 ± 0.69^b	6.3 ± 1.9^b	3.5 ± 0.79^c	1.2 ± 0.71^b	0.69 ± 0.027^b	-

* Calculated based on the amount of Fe(II) oxidized during the following incubation time; ^a 0-73 h, ^b 0-70 h, ^c 0-67 h (Fig. 4.1).
The rates given are mean values $\pm$ SD; n = 2.

Fe(II) oxidation in cell-suspensions of six *Sulfolobus* strains are compared in Fig. 4.1. No further cell growth was observed in cell-suspensions during the experiments. A little decrease in total soluble Fe concentrations was noticed cell-suspensions during Fe(II) oxidation experiments. However, this resulted from precipitation of Fe(III) formed by preceding microbial Fe(II) oxidation. Therefore, decrements in Fe(II) concentrations were regarded as the amount of Fe(II) oxidized to Fe(III), and possible contribution of cells as Fe(II) biosorbent was therefore ignored.

The Fe(II) oxidation rates (mg-Fe/h for comparison between abiotic and biotic Fe(II) oxidations) and specific Fe(II) oxidation rates (mg-Fe/h/cell for comparison between *Sulfolobus* strains) were calculated (using the data from designated hours in Fig. 4.1) and listed in Table 1. Based on the Fe(II) oxidation rates (mg-Fe/h) in cell-suspensions compared with those in cell-free controls, microbial Fe(II) oxidation was detected in all six strains, but to a highly varying extent (Table 4.1). *S. solfataricus* P1, *S. shibatae* B12 and *Sulfolobus* sp. GA1 showed minor to marginal (but still distinguishable) microbial Fe(II) oxidation, compared with the abiotic counterpart. According to the specific Fe(II) oxidation rates (mg-Fe/h/cell), *Sulfolobus* sp. GA1 was the weakest in Fe(II) oxidation, followed by *S. shibatae* B12 and *S. solfataricus* P1 (Table 4.1).

The most effective Fe(II) oxidation was observed in cell-suspensions of *S. metallicus* Kra23, followed by *S. tokodaii* 7 (Fig. 4.1). It should be noted that the final cell density regularly achieved in pre-grown cultures (for preparation of cell-suspensions) of strictly autotrophic *S. metallicus* Kra23 (approximately 2×10^8 cells/mL) was much lower than that of heterotrophic *S. tokodaii* 7 (approximately 5×10^8 cells/mL). Therefore, the specific Fe(II) oxidation rate (mg-Fe/h/cell) was over 4 times greater with *S. metallicus* Kra23 than with *S. tokodaii* 7 (Table 4.1).

S. acidocaldarius 98-3 was less effective Fe(II) oxidizer than *S. metallicus* Kra23 and *S. tokodaii* 7, but clearly more effective than the rest (Fig. 4.1; Table 4.1).

Brock et al (1976) reported Fe(II) oxidation in Yellowstone hot spring waters that included significant populations of *S. acidocaldarius*, but whether or not this observation indeed resulted from Fe(II) oxidation by this specific species was so far unclear. The results here showed that *S. acidocaldarius* 98-3, isolated from the same water source (Brock et al 1972), indeed possesses Fe(II)-oxidizing ability with a

moderate effect (compared to other *Sulfolobus* strains tested). Therefore, it was indicated that this species indeed contributes/contributed Fe(II) oxidation in Yellowstone hot springs.

Bathe and Norris (2007) reported that *S. tokodaii* readily oxidized Fe(II) during heterotrophic growth on yeast extract, but its autotrophic Fe(II) oxidation was much weaker than that by *S. metallicus*. Our results demonstrated that *S. metallicus* Kra23 and *S. tokodaii* 7 oxidize Fe(II) much more readily than the others tested. Since *S. metallicus* is the only known strictly autotrophic type strain in the genus (Huber and Stetter 1991), it is reasonable that this species oxidizes Fe(II) much more effectively than all the others to support its growth.

4.3.2 Comparison of Fe(III) reduction by six *Sulfolobus* strains in growth-uncoupled cell-suspensions under anaerobic / micro-aerobic conditions

Among the six *Sulfolobus* strains tested, only *S. metallicus* Kra23 is known to grow autotrophically on S^0 as energy source (Huber and Stetter 1991). Other five strains grow heterotrophically on glucose as a sole electron donor (Grogan 1989; Grogan et al 1990; Suzuki et al 2002; Masaki et al 2016). The following Fe(III) reduction experiments therefore used S^0 and glucose as electron donor for *S. metallicus* Kra23 and the rest, respectively.

Fe(III) reduction in cell-suspensions of six *Sulfolobus* strains under anaerobic and micro-aerobic conditions are compared in Fig. 4.2 and Fig. 4.3, respectively. No further cell growth was apparent in cell-suspensions during the experiments. The specific Fe(III) reduction rates (mg-Fe/h/ 1×10^{10} cells for anaerobic experiments; mg-Fe/h/ 1.5×10^{10} cells for micro-aerobic experiments) were calculated (using the data from designated hours in Fig. 4.2 and 4.3) and listed in Table 4.2.

S. metallicus Kra23, the strongest Fe(II) oxidizer among the six strains, was shown to be also the most effective Fe(III) reducer under anaerobic conditions (Fig. 4.2a; Table 4.2). When tested micro-aerobically, however, its Fe(III)-reducing ability was almost totally suppressed (Fig. 4.3a; Table 4.2).

S. tokodaii 7 readily reduced Fe(III) under anaerobic conditions with both S^0 and glucose as electron donor (Fig. 4.2b; Table 4.2). Under micro-aerobic conditions,

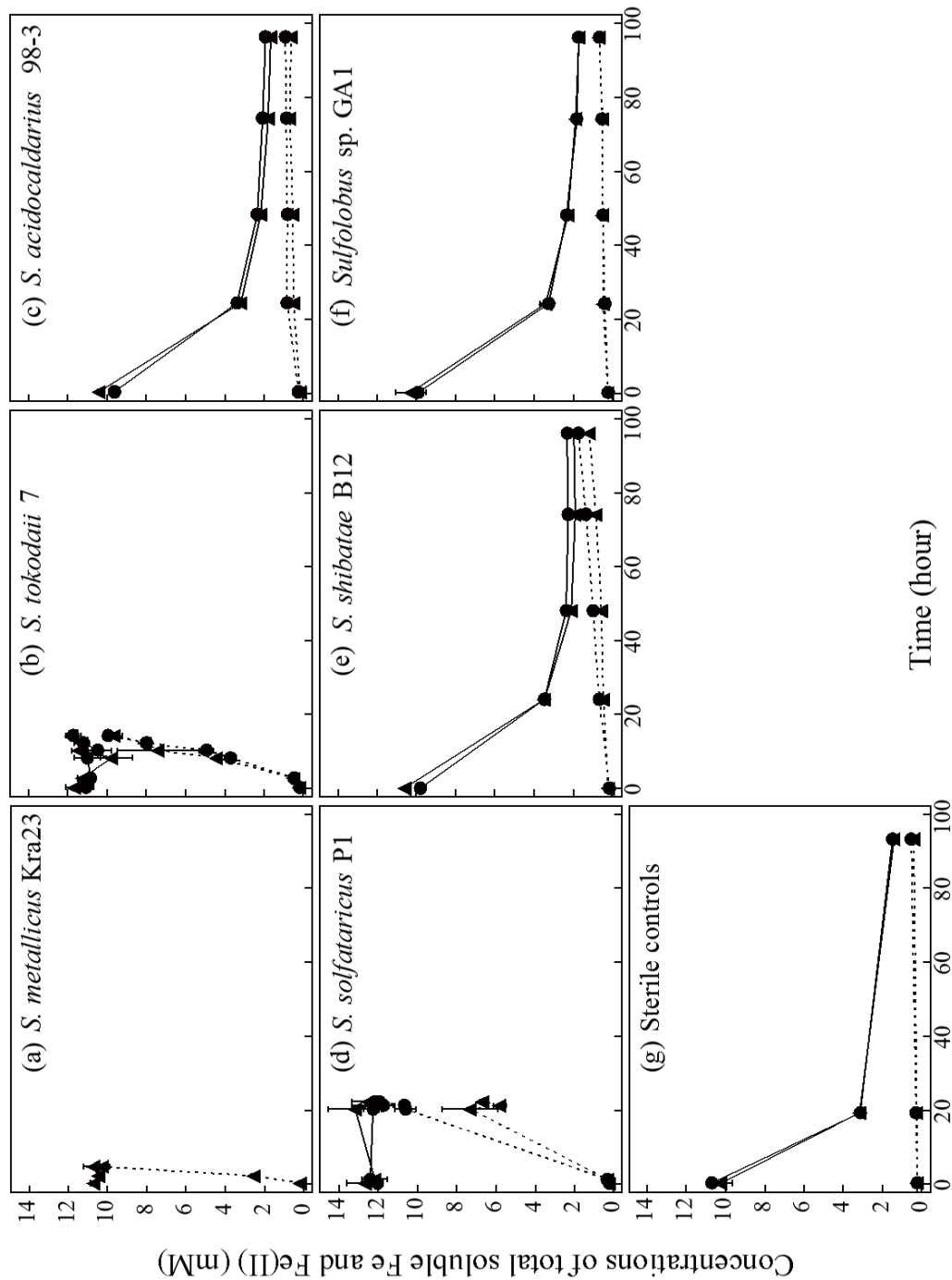


Fig. 4.2 Growth-uncoupled Fe(III) reduction in cell-suspensions of six *Sulfolobus* strains (pH 2.0, 70° C, anaerobic conditions): (a) *S. metallicus* Kra23, (b) *S. tokodaii* 7, (c) *S. acidocaldarius* 98-3, (d) *S. solfataricus* P1, (e) *S. shibatae* B12, (f) *Sulfolobus* sp. GA1, (g) sterile controls. Glucose (●) or elemental sulfur (▲) was used as electron donor. Solid or broken lines indicate concentrations of total soluble Fe or Fe(II), respectively. Data points are mean values from duplicate experiments. Error bars depicting averages are not visible in some cases as these were smaller than the data point symbols.

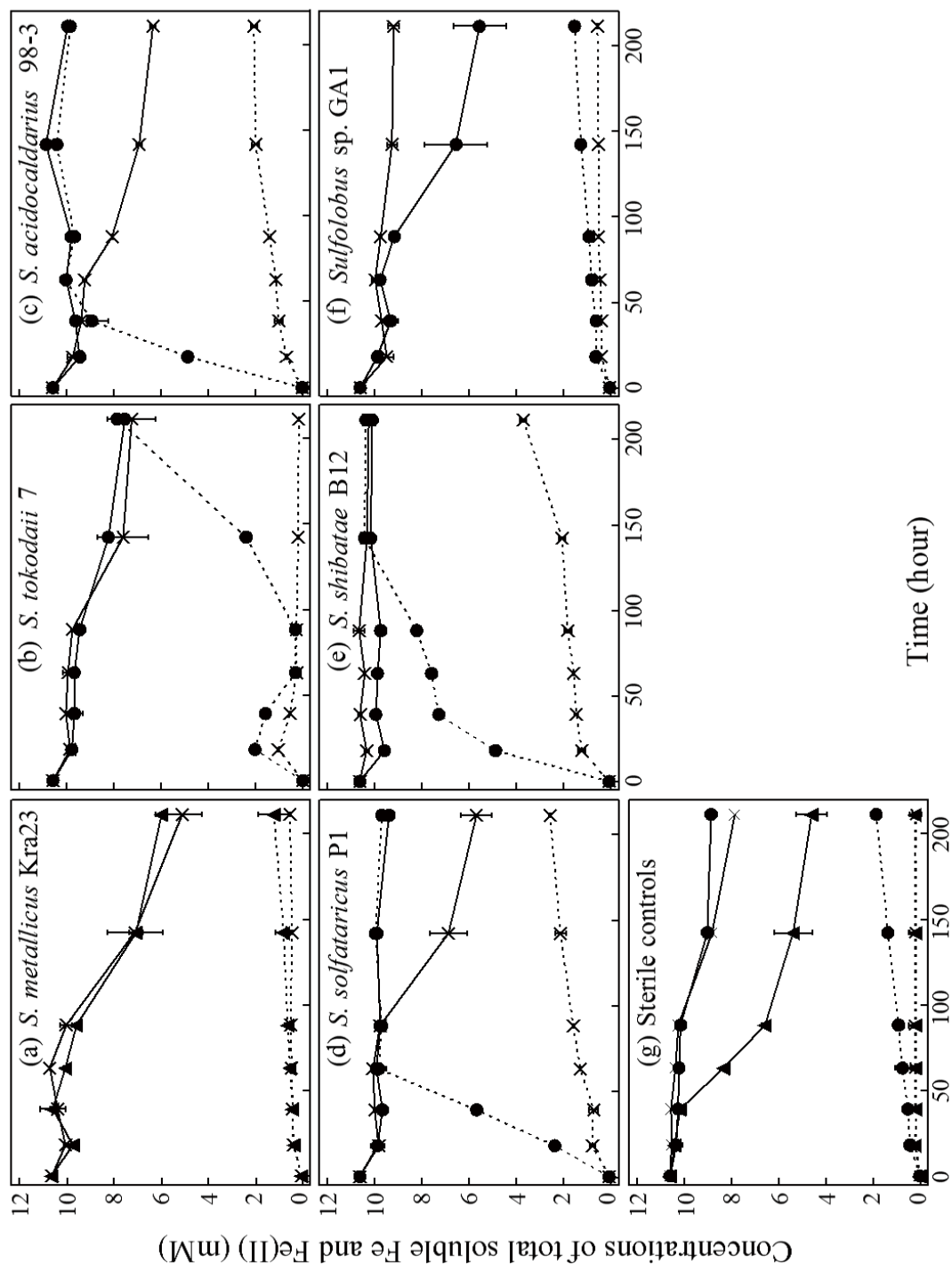


Fig. 4.3 Growth-uncoupled Fe(III) reduction in cell-suspensions of six *Sulfolobus* strains (pH 2.0, 70° C, micro-aerobic conditions): (a) *S. metallicus* Kra23, (b) *S. tokodaii* 7, (c) *S. acidocaldarius* 98-3, (d) *S. solfataricus* P1, (e) *S. shibatae* B12, (f) *S. solfataricus* sp. GA1, (g) sterile controls. Glucose (●) or elemental sulfur (▲) was used as electron donor. Electron-donor-free controls (×) were also prepared. Solid or broken lines indicate concentrations of total soluble Fe or Fe(II), respectively. Data points are mean values from duplicate experiments. Error bars depicting averages are not visible in some cases as these were smaller than the data point symbols.

Table 4.2 Specific Fe(III) reduction rates determined for six *Sulfolobus* strains in growth-uncoupled cell suspensions under anaerobic or micro-aerobic conditions.

Conditions \ <i>Sulfolobus</i> strains		<i>S. metallicus</i> Kra23	<i>S. tokodaii</i> 7	<i>S. acidocaldarius</i> 98-3	<i>S. solfataricus</i> P1	<i>S. shibatae</i> B12	<i>Sulfolobus</i> sp. GA1	Cell-free controls
<u>Specific Fe(III) reduction rates</u> <u>Electron donor</u>								
Anaerobic conditions ($\times 10^{-2}$ mg-Fe/h/ 1×10^{10} cells)	Glucose	-	39 ± 0.31^b	1.9 ± 0.05^c	30 ± 0.12^d	1.5 ± 0.04^c	0.76 ± 0.006^c	0.19 ± 0.02^e
	S ⁰	127 ± 4.6^a	38 ± 1.6^b	1.1 ± 0.01^c	17 ± 0.24^d	1.0 ± 0.006^c	0.94 ± 0.006^c	0.08 ± 0.001^e
Micro-aerobic conditions ($\times 10^{-2}$ mg-Fe/h/ 1.5×10^{10} cells)	Glucose (S ⁰ for Kra23)	1.5 ± 0.02^f	9.4 ± 0.52^f	23 ± 0.01^f	11 ± 0.41^f	22 ± 0.14^f	2.7 ± 0.4^f	$1.9 \pm 0.05^f (0.93 \pm 0.04^f; S^0)$
	None	1.6 ± 0.04^f	4.8 ± 0.01^f	3.1 ± 0.17^f	3.3 ± 0.51^f	5.4 ± 0.72^f	1.6 ± 0.009^f	0.84 ± 0.03^f

* Calculated based on the amount of Fe(II) reduced during the following incubation time; ^a 0-4.5 h, ^b 0-14 h, ^c 0-24 h, ^d 0-22 h, ^e 0-19 h, ^f 0-18 h (Fig. 4.2 and 4.3).
The rates given are mean values $\pm$ SD; n = 2.

however, Fe(III) reduction and Fe(II) oxidation became alternatively noticeable until residual oxygen was consumed, which was later followed solely by Fe(III) reduction (Fig. 4.3b).

S. solfataricus P1 also displayed fairly strong Fe(III)-reducing ability (more readily with glucose than S^0 as electron donor) under anaerobic conditions (Fig. 4.2d; Table 4.2), which was slightly negatively affected by the presence of residual oxygen when glucose was used as electron donor (Fig. 4.3d; Table 4.2).

S. acidocaldarius 98-3 and *S. shibatae* B12 showed the least Fe(III) reduction among the six strains under anaerobic conditions (glucose was the better electron donor than S^0 for Fe(III) reduction in both strains) (Fig. 4.2c, e; Table 4.1). However, Fe(III) reduction by the two strains were greatly facilitated under micro-aerobic conditions, showing the most effective Fe(III) reduction among the six strains (Fig. 4.3c, e; Table 4.2). Again, this was not caused by a cell number increase in the presence of residual oxygen, since there was no further cell growth in cell-suspensions during the experiment.

Although *Sulfolobus* sp. GA1 was initially isolated as Fe(III)-reducing strain (Masaki et al 2016), no clear evidence of Fe(III) reduction was seen in its growth-uncoupled cell-suspensions under both anaerobic and micro-aerobic conditions (Fig. 4.2, 4.3; Table 4.2).

4.3.3 Cell growth-coupled dissimilatory Fe(III) reduction in *Sulfolobus* spp. under anaerobic conditions

In order to investigate whether or not Fe(III) can serve as sole electron acceptor for *Sulfolobus* growth, correlation between cell density and Fe(III) reduction was monitored after inoculation (at 1×10^7 cells/mL) under strictly anaerobic conditions (Fig. 4.4).

Linear correlations between cell densities and the amounts of Fe(III) reduced were observed in *S. tokodaii* 7 (Fig. 4.4b), *S. solfataricus* P1 (Fig. 4.4d), *S. shibatae* B12 (Fig. 4.4e), and *Sulfolobus* sp. GA1 (Fig. 4.4f): This clearly indicates that these 4 strains are capable of anaerobic growth using glucose and Fe(III) as sole electron donor and acceptor, respectively.

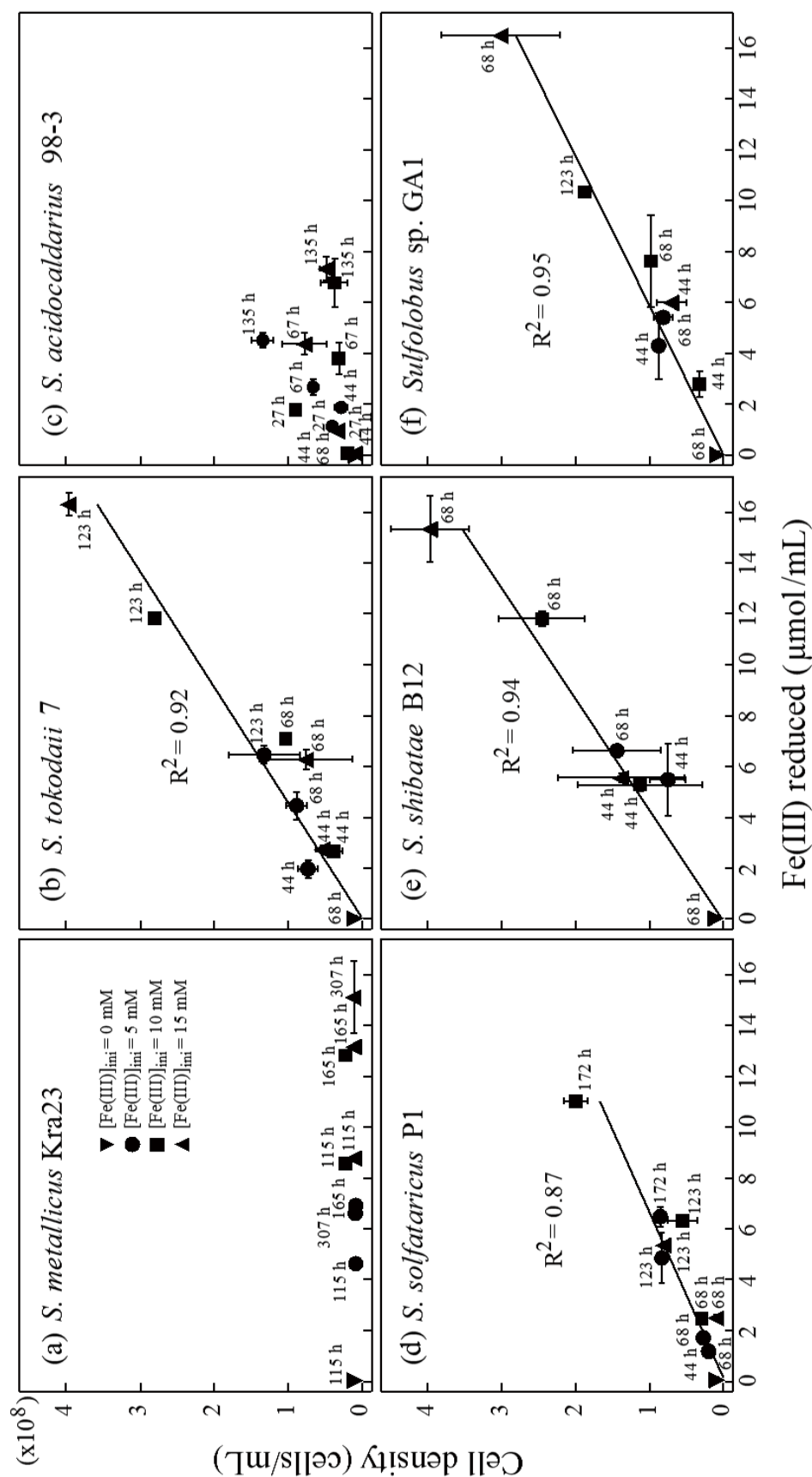


Fig. 4.4 Correlation between anaerobic cell growth and the amount of Fe(III) reduced by six *Sulfolobus* strains (pH 2.0, 70° C): (a) *S. metallicus* Kra23, (b) *S. tokodaii* 7, (c) *S. acidocaldarius* 98-3, (d) *S. solfataricus* P1, (e) *S. shibatae* B12, (f) *Sulfolobus* sp. GA1. The initial Fe(III) concentration used were 0 (∇), 5 ($\bullet$), 10 ($\blacksquare$), or 15 ($\blacktriangle$) mM. The number indicated for each plot indicates the time for sampling. Data points are mean values from duplicate experiments. Error bars depicting averages are not visible in some cases as these were smaller than the data point symbols.

In the case of other two strains, *S. metallicus* Kra23 and *S. acidocaldarius* 98-3, Fe(III) reduction did occur by the existing cells, but no correlation between cell growth and Fe(III) reduction was found (Fig. 4.4a, c).

Interestingly, the microbial capability of growth-coupled Fe(III) respiration did not necessarily correspond to efficient Fe(III) reduction in its growth-uncoupled cell-suspensions. The results from a series of Fe(III) reduction experiments revealed that all tested *Sulfolobus* spp. basically possess Fe(III) reduction capabilities, whose functions and response to the oxygen level, however, differ significantly between the strains as summarized as follows: (i) *S. metallicus* Kra23: The strongest Fe(III) reducer among those tested under anaerobic conditions; incapable of anaerobic growth using S^0 and Fe(III) as sole electron donor and acceptor, respectively; residual oxygen significantly suppresses its Fe(III) reduction. (ii) *S. tokodaii* 7: Readily reduces Fe(III) under anaerobic conditions; its Fe(II) oxidation can overtake Fe(III) reduction in the presence of residual oxygen, exhibiting apparent alternate switch on/off of Fe(III) reduction and Fe(II) oxidation; capable of anaerobic growth using glucose and Fe(III) as sole electron donor and acceptor, respectively. (iii) *S. solfataricus* P1: Fairly readily reduces Fe(III) under anaerobic conditions; Fe(III) reduction slightly negatively affected by the residual oxygen; capable of anaerobic growth using glucose and Fe(III) as sole electron donor and acceptor, respectively. (iv) *S. acidocaldarius* 98-3: One of the weakest Fe(III) reducer among those tested under anaerobic conditions, whilst micro-aerobic conditions significantly accelerate its Fe(III)-reducing ability; no anaerobic growth observed using glucose and Fe(III) as sole electron donor and acceptor, respectively. (v) *S. shibatae* B12: Another one of the weakest Fe(III) reducer among those tested under anaerobic conditions, whilst micro-aerobic conditions significantly accelerate its Fe(III)-reducing ability; capable of anaerobic growth using Fe(III) as sole electron acceptor (glucose as electron donor). (vi) *Sulfolobus* sp. GA1: Fe(III) reduction was observed only as growth-coupled anaerobic Fe(III) respiration. No clear evidence was found for Fe(III) reduction in growth-uncoupled cell-suspensions both under anaerobic and micro-aerobic conditions. Although *Sulfolobus* sp. GA1 is most closely related to *S. shibatae* B12 (with 99.7% 16S rRNA gene sequence identity; Masaki et al 2016), their Fe oxido-reduction behaviors were not always similar to each other. Response of Fe(III) reduction to residual oxygen (micro-aerobic conditions) was significantly

different between the two strains.

Comparably high standard redox potential of Fe(III)/Fe(II) (+0.76V; pH 2.0) to that of $1/2\text{O}_2/\text{H}_2\text{O}$ (+0.82V) makes iron favorable alternative electron acceptor for acidophiles under anaerobic conditions (Madigan and Martinko, 2006). *Sulfolobus* strains responded diversely to the residual oxygen in their Fe(III) reduction activity, likely reflecting the different mechanisms involved in the electron transport chain for their Fe(III) reduction.

4.3.4 Comparison of Cr(VI) reduction by six *Sulfolobus* strains in growth-uncoupled cell-suspensions under micro-aerobic conditions

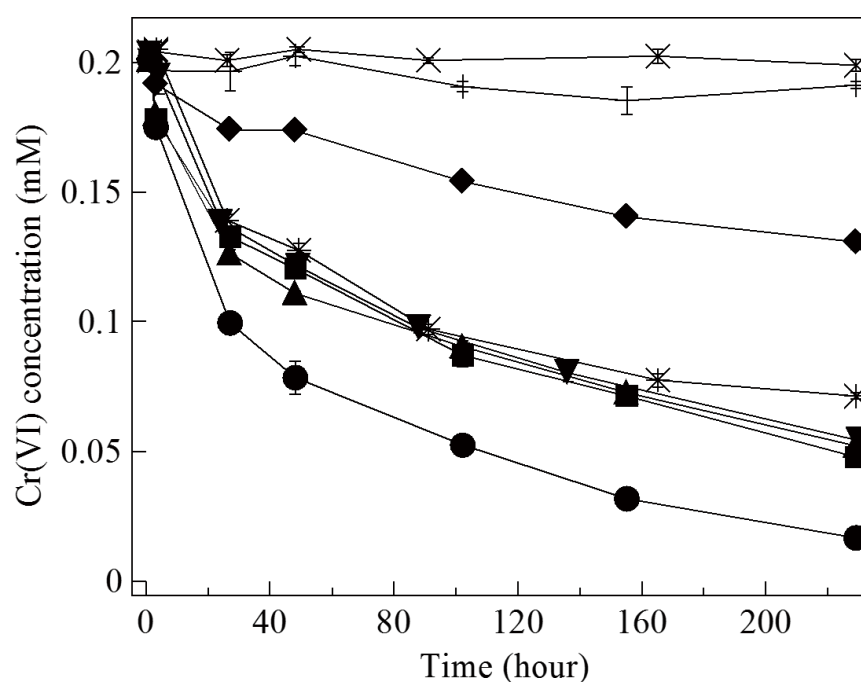


Fig. 4.5 Cr(VI) reduction in cell-suspensions of six *Sulfolobus* strains (pH 2.0, 70°C, micro-aerobic conditions): *S. metallicus* Kra23 (*); *S. tokodaii* 7 (■); *S. acidocaldarius* 98-3 (◆); *S. solfataricus* P1 (▼); *S. shibatae* B12 (●); *Sulfolobus* sp. GA1 (▲); sterile controls (+; glucose, x; elemental sulfur). Data points are mean values from duplicate experiments. Error bars depicting averages are not visible in some cases as these were smaller than the data point symbols.

Following the previous observation that Fe(III)-reducing *Sulfolobus* sp. GA1 is also capable of Cr(VI) reduction to Cr(III) (Masaki et al 2016), this study tested six *Sulfolobus* strains to investigate whether or not Cr(VI)-reducing ability is also widely found across different *Sulfolobus* species.

Since no changes in total soluble Cr concentration were observed throughout the experiment (data not shown), Cr(VI) reduced by *Sulfolobus* strains remained soluble mostly in the form of Cr(III). Liquid media did not contain any trace of iron and no noticeable abiotic reduction of Cr(VI) was observed in sterile controls (Fig. 4.5).

All six strains were found to reduce Cr(VI), though to a different extent (Fig. 4.5) and with different specific Cr(VI) reduction rates (Table 4.3). Interestingly, the trend in degree of Fe(III) reduction by six strains (under the same micro-aerobic conditions) did not necessarily correspond to that of Cr(VI) reduction: e.g., *S. acidocaldarius* 98-3 and *S. shibatae* B12 showed the highest Fe(III) reduction under micro-aerobic conditions (Fig. 3c, e, respectively). Nonetheless, the former was the least effective Cr(VI) reducer while the latter was the most effective among the six strains.

Oxido-reduction of metal species are often mediated by cytochromes in electron transport chains: In the case of acidophilic bacteria, *c*-type cytochromes in cell membranes were found to be involved in anaerobic Fe(III) reduction by *At. ferrooxidans* (Ohmura et al 2002). Cr(VI) reduction by Fe(III)-respiring acidophile *A. cryptum* was also reported to involve *c*-type cytochromes (Magnuson et al 2010). In the case of Fe(III)-reducing neutrophiles, *c*-type cytochromes were found to function in Fe(III) reduction by *Geobacter sulfurreducens* (Magnuson et al 2000) and both Fe(III) and Cr(VI) reduction by *Shewanella putrefaciens* (Beliaev et al 2001). In the latter bacterium, Cr(VI) reductase was reported to be distinct from Fe(III) reductase, and was not irreversibly inhibited by oxygen (Myers et al 2000).

The fact that the trend in Fe(III) reduction by six *Sulfolobus* strains does not always match that in Cr(VI) reduction imply that different mechanisms may be involved in microbial reduction of the two metals in *Sulfolobus* spp.

Table 4.3 Specific Cr(VI) reduction rates determined for six *Sulfolobus* strains in growth-uncoupled cell suspensions under micro-aerobic condition.

<i>Sulfolobus</i> strains	<i>S. metallicus</i> Kra23	<i>S. tokodaii</i> 7	<i>S. acidocaldarius</i> 98-3	<i>S. solfataricus</i> P1	<i>S. shibatae</i> B12	<i>Sulfolobus</i> sp. GA1	Cell-free controls
Specific Cr(VI) reduction rates* ($\times 10^{-2}$ mg-Cr/h/ 5.0×10^{10} cells)	0.64 ± 0.024^a	0.68 ± 0.01^b	0.27 ± 0.009^b	0.73 ± 0.01^c	1.0 ± 0.01^b	0.75 ± 0.0008^b	0.057 ± 0.05^b (0.034 ± 0.017^a ; S^0)

* Calculated based on the amount of Cr(VI) reduced during following incubation time; ^a 0-25 h, ^b 0-26 h, ^c 0-23 h. The rates given are mean values $\pm$ SD; n = 2.

4.3.5 Cr(VI) reduction by high density cell-suspensions of *Sulfolobus* sp. and *S. shibatae* B12, and the following Cr immobilization through biosorption

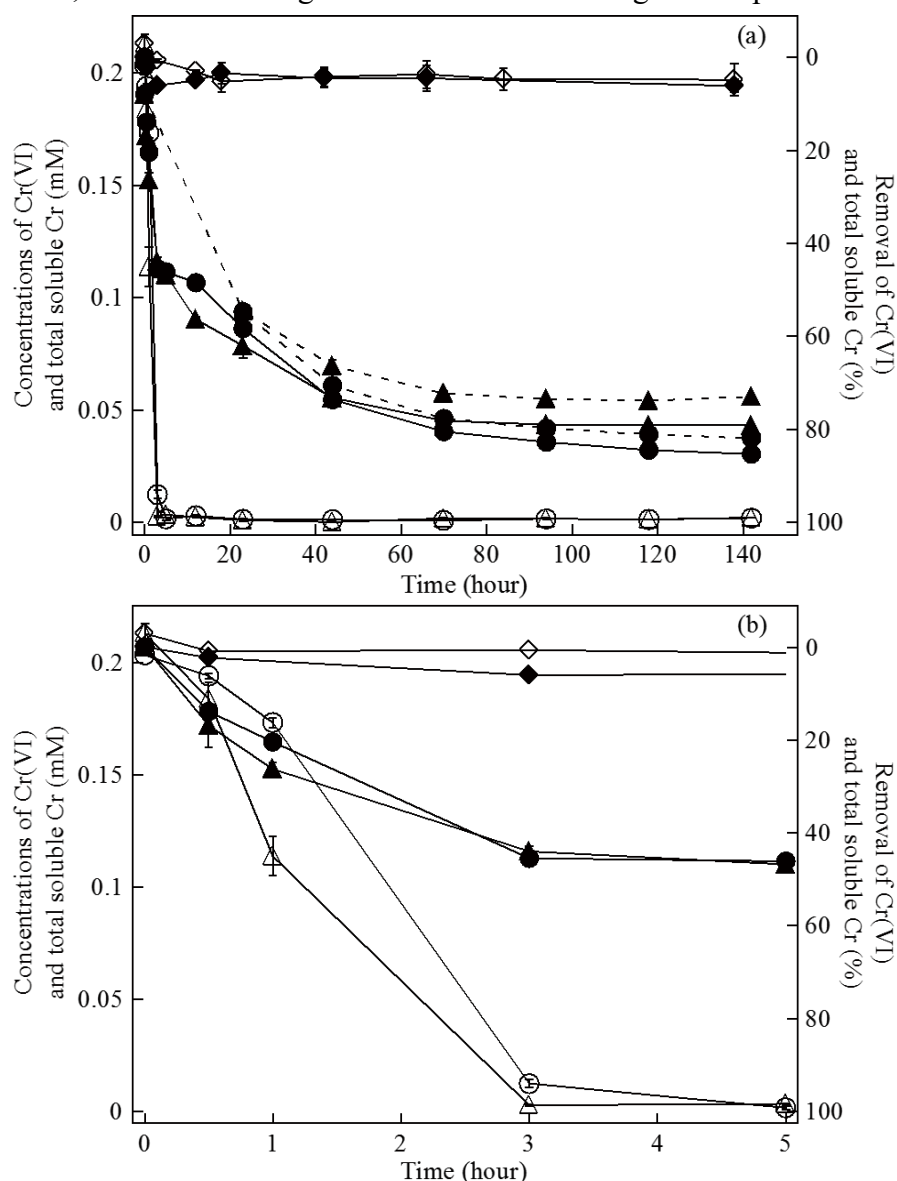


Fig. 4.6 (a) Cr(VI) reduction (open symbols) and Cr immobilization (solid symbols) in cell-suspensions of high density cell-suspensions of *Sulfolobus* sp. GA1 (▲, △) and *S. shibatae* B12 (●, ○) and sterile controls (◆, ◇) under micro-aerobic conditions at pH 2.5 (70°C) in the presence of 1 mM glucose. High density cell-suspensions (1.0×10^{10} cells/mL) were incubated with 0.2 mM Cr(VI) (solid lines) or with 0.2 mM Cr(III) (broken lines). The sterile control was incubated with 0.2 mM Cr(VI) (solid line). (b) The data until day 5 are shown. Data points are mean values from duplicate cultures; error bars depicting averages are not visible as these were smaller than the data point symbols in all cases.

Since Cr immobilization was not confirmed when using cell-suspensions of 1×10^9 cells/mL in the previous experiment, 10-fold higher density cell-suspensions were used in this experiment to improve Cr immobilization through biosorption and also to increase the level of Cr(VI) reduction. The increase in cell density resulted in improvement of microbial Cr(VI) reduction and Cr immobilization in both *Sulfolobus* sp. GA1 and *S. shibatae* B12 at pH 2.5, while noticeable abiotic Cr(VI) reduction and Cr immobilization were not observed in sterile controls throughout the experiment (Fig. 4.6a). Significant drops in the concentration of soluble Cr(VI) were observed in high density cell-suspensions of both strains (Fig. 4.6b). The cell-suspensions of *Sulfolobus* sp. GA1 and *S. shibatae* B12 readily reduced 99% and 94% of the Cr(VI) respectively (initial Cr(VI) concentration of 0.2 mM), within first 3 h, accompanied by sudden decreases in the concentration of total soluble Cr; 44% in the former, 46% in the latter (Fig. 4.6a). Both cell-suspensions of the strains achieved approximately 99% reduction of Cr(VI) after a total of 5 h. The specific rates of Cr(VI) reduction using 1×10^{10} cells/mL cell-suspensions were calculated to be 0.14×10^{-10} and 0.21×10^{-10} mg Cr/h/cell in *Sulfolobus* sp. GA1 and *S. shibatae* B12, respectively. Both specific rates were approximately double those observed using 1.0×10^9 cells/mL suspensions. Since the same initial Cr(VI) concentration was used in both experiments using 1×10^9 or 1×10^{10} cells/mL cell-suspensions, this result may be due to the chemical equilibrium of the Cr(VI) reduction reaction: Reduction rates of Cr(VI) were accelerated using a higher density cell-suspension, since an increase in cell density, here, increases the concentration of the reducing agent. The Cr(VI) concentrations in solution at the end of the experiment were only 2.1 and 1.9 μM in *Sulfolobus* sp. GA1 and *S. shibatae* B12, respectively, almost meeting the environmental standard for Cr(VI) of 0.96 μM (0.05 mg/L;

[http://www.who.int/water_sanitation_health/dwq/chemicals/chromium.pdf#search='hexavalent+chromium+WHO+drinking+water'\)](http://www.who.int/water_sanitation_health/dwq/chemicals/chromium.pdf#search='hexavalent+chromium+WHO+drinking+water'))).

The concentration of total soluble Cr continuously decreased in the cell-suspensions, resulting in 79% and 85% loss of total soluble Cr from solution phase within 142 h by *Sulfolobus* sp. GA1 and *S. shibatae* B12, respectively. Chromium immobilization via biosorption onto the cell surface was shown by the absence of precipitates throughout the experiment. When 0.2 mM Cr(III), instead of Cr(VI), was

initially added to the high density cell-suspensions, both strains were found to adsorb Cr; 71% of the total soluble Cr for *Sulfolobus* sp. GA1 and 80% for *S. shibatae* B12, within 142 h. The biosorption behaviors were quite similar to those when Cr(VI) was initially added.

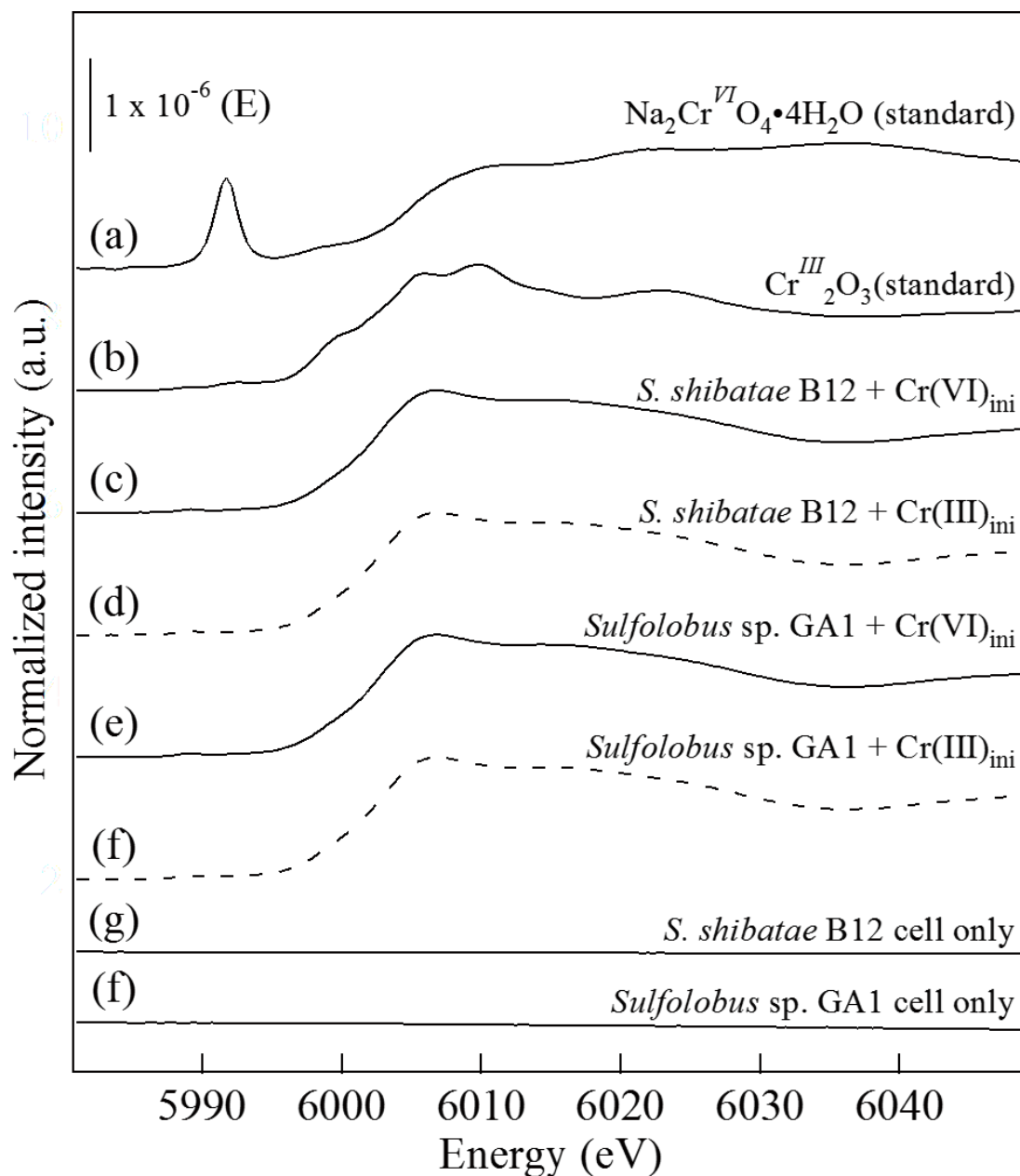


Fig. 4.7 Normalized XANES spectra at the Cr K-edge for Cr standards (a, b), and for cell pellets of *Sulfolobus* sp. GA1 and *S. shibatae* B12 after 166 h incubation with Cr(VI) (c and e, respectively), Cr(III) (d and f, respectively), or without Cr (g and h, respectively).

Given that the adsorbed state of Cr, i.e. as Cr(VI) or Cr(III), is an important factor to evaluate Cr biosorption, XANES analysis was conducted with Cr(VI) and Cr(III) standards ($\text{Na}_2\text{CrO}_4 \cdot 4\text{H}_2\text{O}$ and Cr_2O_3 , respectively), cells of both strains incubated with Cr(VI) or Cr(III), and cells of both strains from Cr-free cultures, in order to investigate the oxidation state of Cr adsorbed onto the cell surface. Absorption edges of the Cr K-edge were observed for cells of both strains incubated with Cr(VI) or Cr(III), but not for the Cr-free cells of both strains, indicating that the Cr immobilization in the Cr(VI) reduction experiment was due to biosorption onto the cell surface of *Sulfolobus* sp. GA1 and *S. shibatae* B12 (Fig. 4.7). Furthermore, cells of both strains incubated with Cr(III), and even those incubated with Cr(VI), were found to immobilize only Cr(III) through biosorption, as evidenced by XANES spectra of the samples lacking a distinct pre-edge peak at 5,992-5,993 eV that would arise from the presence of Cr(VI) (Peterson et al 1996, Sparks et al 2002).

The zeta-potential of *S. acidocaldarius* cells has been determined to be -0.7 mV at pH 2.0 and -0.4 mV at pH 3.0 (Vitaya et al 1991). Moreover, *S. acidocaldarius* 98-3 has been reported to adsorb uranium ions (UO_2^{2+}), onto the cell surface at acidic pHs (Reitz et al 2010). These data imply that, as well as *S. acidocaldarius*, both *Sulfolobus* sp. GA1 and *S. shibatae* B12 possess negatively-charged cell surfaces. It has been reported that Cr(VI) and Cr(III) supposedly exist in solution as HCrO_4^- and $\text{Cr}(\text{H}_2\text{O})_6^{3+}$, respectively, at pH 2.5 (Poulopoulou et al 1997, Kritayakornupong et al 2004, Cornelis et al 2005, Hawley et al 2005). *Sulfolobus* sp. GA1 and *S. shibatae* B12 may electrostatically adsorb cations, i.e. $\text{Cr}(\text{H}_2\text{O})_6^{3+}$, but not anions, i.e. HCrO_4^- , onto their negatively-charged cell surfaces. By extension, other strains of *Sulfolobus* may also adsorb externally-added Cr(III) and microbially-reduced Cr(III), and even other cations formed by heavy metals.

Studies on microbiological reduction of Cr(VI) and the following immobilization of Cr using extreme acidophiles have previously, as far as we know, been limited to *A. cryptum* JF-5 and *Acidocella* (*Ac.*) *aromatica* PFBC, Fe(III)-reducing acidophiles (Cummings et al 2007, Masaki et al 2015). *A. cryptum* JF-5 and *Ac. aromatica* PFBC have both been reported to reduce Cr(VI) to Cr(III), and subsequently, to immobilize Cr only in the form of Cr(III) through biosorption onto the cell surface. Here, *Sulfolobus* sp. GA1 and *S. shibatae* B12 showed similar behavior in Cr(VI) reduction

and Cr immobilization. Comparing the Cr(VI) reduction capabilities of *S. shibatae* B12 and *Ac. aromatica* PFBC, the specific rate of Cr(VI) reduction by the former (0.21×10^{-10} mg Cr/h/cell) was much higher than that by the latter (0.04×10^{-10} mg Cr/h/cell; under the same conditions, except for the addition of fructose instead of glucose, and a reaction temperature of 30°C; Masaki et al 2015). When Cr(VI) was added initially, 85% of the total soluble Cr was adsorbed onto the cell surface of *S. shibatae* B12 (solely as Cr(III)), whereas only 38% was adsorbed by *Ac. aromatica* PFBC cells. The reason for this difference may be the adsorption capacity of the strains: Zeta-potential values of cells generally vary depending on the proportions of a variety of functional groups on the cell surface, including carboxyl, phosphate, and amino groups. For *Ac. aromatica* PFBC, when Cr(III) was added initially, the amount of Cr(III) adsorbed onto the cell surface was reported to be only 5%. Masaki et al. (2015) concluded that microbially-reduced Cr(III), but not externally-provided Cr(III), was readily immobilized via biosorption by *Ac. aromatica* PFBC cells. In contrast, the initial valence state of Cr did not affect the Cr immobilization capability of *S. shibatae* B12 cells. This suggests that cells of *Sulfolobus* sp. GA1 and *S. shibatae* B12 may adsorb Cr(III) via a different mechanism from that of *Ac. aromatica* PFBC.

4.4 Conclusions

This study clarified the presence and the degree of Fe oxido-reduction abilities in six representative *Sulfolobus* strains (*S. metallicus* Kra23; *S. tokodaii* 7; *S. acidocaldarius* 98-3; *S. solfataricus* P1; *S. shibatae* B12; *Sulfolobus* sp. GA1). The degree of Fe(II)-oxidizing abilities were found to differ largely between the strains. Four strains (*S. tokodaii* 7, *S. solfataricus* P1, *S. shibatae* B12 and *Sulfolobus* sp. GA1) were capable of anaerobic growth via Fe(III) respiration. All strains other than *Sulfolobus* sp. GA1 displayed Fe(III)-reducing abilities in growth-uncoupled cell-suspensions. Nonetheless, Fe(III)-reducing ability in each strain responded significantly differently to the oxygen level. Highly-toxic Cr(VI) was also reduced in growth-uncoupled cell-suspensions of all strains, though to a varying degree. To summarize, it was revealed that *Sulfolobus* spp. play important roles in Fe redox transformations in natural environments. The degree and mechanism of Fe oxido-reduction reactions were found versatile between different species. The trend of Fe(III) reduction in six strains did not correspond to that of Cr(VI), suggesting different mechanisms being involved in Fe(III) and Cr(VI) reduction in *Sulfolobus* spp. For being a major member in the acidophile community, Fe redox transformation abilities in the genus *Sulfolobus* was yet largely ambiguous. The results of this study provide comprehensive understandings over the genus *Sulfolobus* on its Fe redox transformation abilities and the potential utility of the *Sulfolobus* spp. for biohydrometallurgical applications.

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The results in this chapter were partially cooperated by Mr. Katsutoshi Tsutsumi (master 2 student in fiscal year 2016).

Chapter 5

Improvement of Ni recovery via reductive bioleaching of limonite ore using Fe(III)-reducing acidophiles

Abstract

Dissolution of laterite ore, especially of limonite ore, via reductive bioleaching using Fe(III)-reducing acidophiles was investigated for maximization of Ni recovery. Screening test of useful Fe(III)-reducing acidophiles resulted in selection of *S. tokodaii* 7 and *Sb. acidophilus* YTF1 from thermophiles and moderately thermophiles tested, respectively. Due to increase in pH caused by Ca contents in the Indonesian limonite ore used in this study, citrate washed-limonite ore was used for reductive bioleaching. Extraction of Ni was found to associate with Fe dissolution in cell suspensions and inoculated cultures of the selected two strains. Owing to microbial Fe(III) reduction, solution redox potential was maintained as low as 200 to 300 mV (vs. SHE) during the reductive bioleaching tests. Fe dissolution was highly correlated to dissolutions of Ni and Al, but not to that of Mg and Ca, indicating that Ni and Al associated with goethite phase in the Indonesian limonite ore. One of the limit factors to Ni extraction was found to be a decrease in solubility capacity of total soluble Fe at higher pHs: The reaction speed of goethite dissolution declined due to a decrease in Fe solubility capacity in leachate, causing lower Ni extraction efficiency. In order to increase Ni recovery percentage, reductive bioleaching was repeated twice or three times with a certain initial pH values. The maximum Ni extraction (within 30 days) of 55 or 41% were marked at the end of the 3rd step using cell suspensions of *S. tokodaii* 7 or *Sb. acidophilus* YTF1, respectively. Overall, Ni extraction via Fe dissolution from the Indonesian limonite ore was found to be sensitive to solution redox potential levels, total soluble Fe concentrations, and solution pHs.

5.1 Introduction

Ni laterite is divided into two types; limonite (mainly consisting of goethite (FeOOH)) and saprolite (known as serpentine ((Ni, Mg, Fe)₃Si₂O₅(OH)₄) rich laterite). Recovery Ni from laterite ore has been studied, and its improvement has been reported via organic/inorganic acid leaching, and further Ni extraction especially from saprolitic laterite, using citrate through Ni-citrate chelation was reported (Tzeferis et al 1994, Valix et al 2001a, 2001b, Le et al 2006, Coto et al 2008, Behera et al 2011, Astuti et al 2015, 2016a, 2016b).

Since around 2010, a microbiological approach, reductive bioleaching using Fe(III)-reducing acidophiles, has newly been proposed for Ni extraction from limonitic ores (Hallberg et al 2011, Johnson 2012). They have suggested a dissolution mechanism of goethite mineral via reductive bioleaching as follows; (i) acid dissolution of goethite via chemical equilibrium of Fe(III) in between solid and soluble phases (Eq. 5.1), and (ii) reduction of soluble Fe(III) to Fe(II) by Fe(III)-reducing acidophiles (Eq. 5.2), promoting dissolution of goethite, followed by extraction of Ni ions. Scheme of its dissolution mechanism is shown in Fig. 5.1.

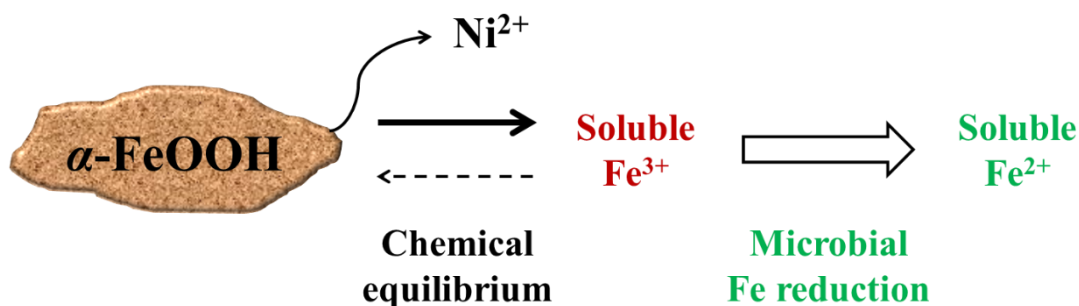


Fig. 5.1 Scheme of reductive bioleaching of goethite for Ni extraction.

5.2 Section 1 Screening of Fe(III)-reducing acidophiles among mesophiles, moderate thermophiles, and thermophiles via capability of soluble Fe(III) reduction and synthesized goethite dissolution under micro-aerobic conditions

5.2.1 Materials and methods

5.2.1.1 Reduction of soluble Fe(III) by cell suspensions of acidophilic microorganisms under micro-aerobic conditions

Cells of the fourteen strains in Table 5.1 were pre-grown, harvested in the late exponential phase by centrifugation ($12,000 \times g$, 10 min, 40°C), washed twice with HBS medium (pH 2.0 with H_2SO_4). To evaluate microbial Fe(III) reduction under micro-aerobic conditions, each of the strain was re-suspended into 15 mL of HBS medium (pH 2.0 with H_2SO_4) containing 10 mM Fe(III) and 1 mM glucose (1 mM fructose, glycerol, or 0.01% (w/v) elemental sulfur, instead of glucose, were added for strains PFBC, T23, or Kra23) with a final cell density of 1×10^9 cells/mL. To establish micro-aerobic conditions, 15-mL Falcon tubes containing the above 15 mL reaction medium were maintained at 30 (mesophiles), 45 (moderate thermophiles), 60 or 70°C (thermophiles) without shaking. Samples were regularly taken after compensation of water evaporated with pure water. Concentration of Fe(II) was monitored. All the experiments were carried out in duplicate.

5.2.1.2 Dissolution of synthesized goethite via reductive bioleaching using microbial Fe(III) reduction by acidophiles under micro-aerobic conditions

Cells of the fourteen strains in Table 5.1 were pre-grown, harvested in the late exponential phase by centrifugation ($12,000 \times g$, 10 min, 40°C), washed twice with HBS medium (pH 2.0 with H_2SO_4). To evaluate microbial Fe(III) reduction under micro-aerobic conditions, each of the strain was re-suspended into 15 mL of HBS medium (pH 2.0 with H_2SO_4) containing 0.2% (w/v) goethite, 0.01% (w/v) yeast extract, and 10 mM glucose (1 mM fructose, glycerol, or 0.01% (w/v) elemental sulfur, instead of glucose, were added for strains PFBC, T23, or Kra23) with a final cell density of 1×10^9 cells/mL. To establish micro-aerobic conditions, 15-mL Falcon tubes containing the above 15 mL reaction medium were maintained at 30 (mesophiles), 45 (moderate thermophiles), 60, or 70°C (thermophiles) without shaking. Samples were

regularly taken after compensation of water evaporated with pure water. Concentrations of Fe(II) and total soluble Fe were monitored. All the experiments were carried out in duplicate.

Table 5.1 Acidophilic microorganisms used for soluble Fe(III) reduction test.

	Microorganisms and sterile controls	Symbols	Temp.	e ⁻ donor
Mesophiles	<i>Acidiphilium</i> sp. SJH	●	30°C	10 mM Glucose
	<i>Ac. aromatica</i> PFBC	▲	30°C	10 mM Fructose
	<i>Ferrimicrobium</i> sp. T23	■	30°C	10 mM Glycerol
	Sterile controls	○	30°C	10 mM Glucose
	Sterile controls	△	30°C	10 mM Fructose
	Sterile controls	□	30°C	10 mM Glycerol
Moderate thermophiles	<i>Ali. acidiphilus</i> TA-67	■	45°C	10 mM Glucose
	<i>Ali. acidoterrestris</i> GD3B	▲	45°C	10 mM Glucose
	<i>Sb. acidophilus</i> YTF1	●	45°C	10 mM Glucose
	<i>Acidiplasma</i> sp. Fv-Ap	◆	45°C	10 mM Glucose
	Sterile controls	○	45°C	10 mM Glucose
Thermophiles	<i>Ali. acidocaldarius</i> subsp. <i>acidocaldarius</i> 104-IA	▲	60°C	10 mM Glucose
	<i>S. shibatae</i> B12	●	70°C	10 mM Glucose
	<i>S. tokodaii</i> 7	■	70°C	10 mM Glucose
	<i>Sulfolobus</i> sp. GA1	*	70°C	10 mM Glucose
	<i>S. acidocaldarius</i> 98-3	+	70°C	10 mM Glucose
	<i>S. solfataricus</i> P1	▼	70°C	10 mM Glucose
	<i>S. metallicus</i> Kra23	◆	70°C	0.01% (w/v) S ⁰
	Sterile controls	○	70°C	10 mM Glucose
	Sterile controls	◇	70°C	0.01% (w/v) S ⁰

5.2.2 Results and discussion

5.2.2.1 Reduction of soluble Fe(III) by cell suspensions of acidophilic microorganisms under micro-aerobic conditions

Microbial reduction of soluble Fe(III) was obviously confirmed in cell suspensions of *Acidiphilium* sp. SJH (30°C), *Ferrimicrobium* sp. T23 (30°C), *Sb. acidophilus* YTF1 (45°C), *S. shibatae* B12 (70°C), *S. tokodaii* 7 (70°C), *S. acidocaldarius* 98-3 (70°C), and *S. solfataricus* P1 (70°C), whereas no abiotic reduction of soluble Fe(III) was observed in sterile controls. In cell suspension of *S. tokodaii* 7, microbial Fe(II) oxidation behavior was observed after an initial microbial Fe(III) reduction due to its strong Fe(II)-oxidizing capability. After, its Fe(II) oxidation, microbial Fe(III) reduction was solely activated. Among thermophiles tested, *S. acidocaldarius* 98-3 was found to show the efficient reduction capability of soluble Fe(III).

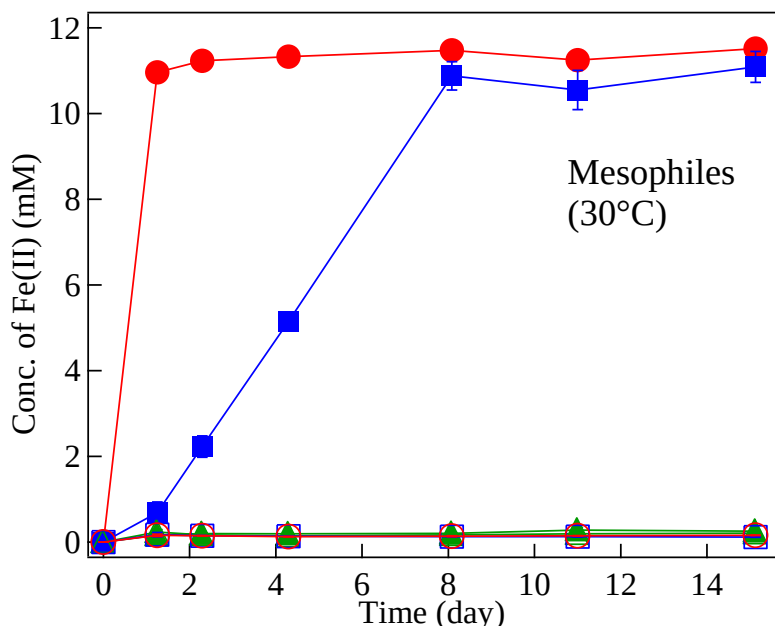


Fig. 5.2 Changes in the concentration of Fe(II) in cell suspensions of *Acidiphilium* sp. SJH (●), *Ac. aromatica* PFBC (▲), and *Ferrimicrobium* sp. T23 (■), and sterile controls with glucose (○), fructose (△), and glycerol (□) at 30°C.

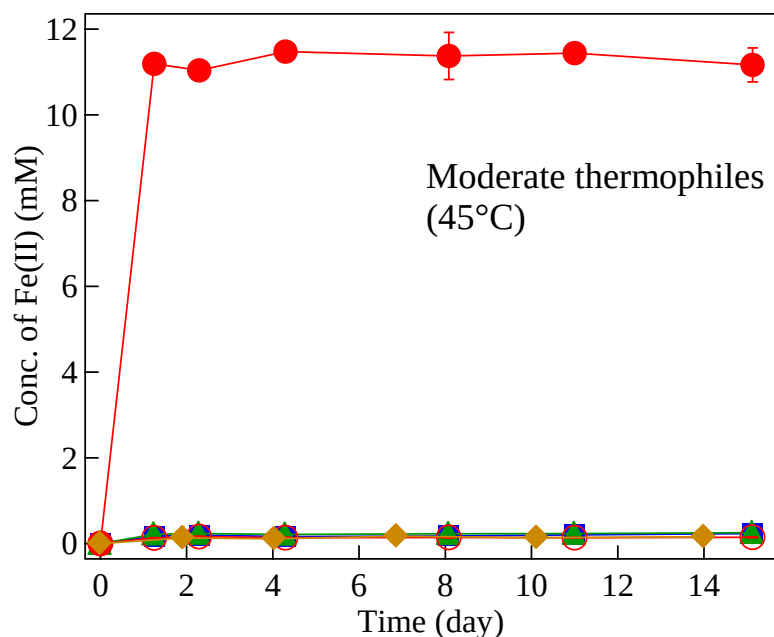


Fig. 5.3 Changes in the concentration of Fe(II) in cell suspensions of *Sb. acidophilus* YTF1 (●), *Ali. acidiphilus* TA-67 (■), *Ali. acidoterrestris* GD3B (▲), and *Acidiplasma* sp. Fv-Ap (◆) and sterile controls with glucose (○) at 45°C.

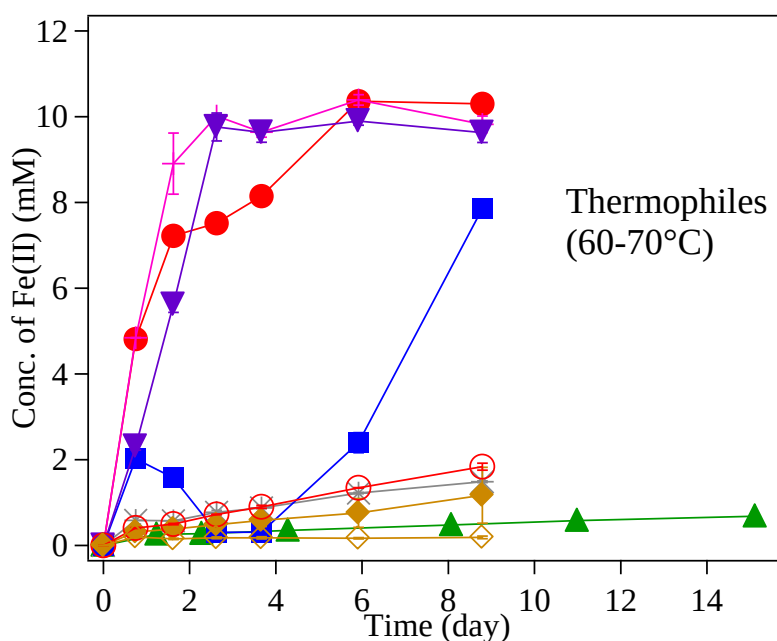


Fig. 5.4 Changes in the concentration of Fe(II) in cell suspensions of *S. shibatae* B12 (●), *S. tokodaii* 7 (■), *S. metallicus* Kra23 (◆), *S. acidocaldarius* 98-3 (+), *S. solfataricus* P1 (▼), *Sulfolobus* sp. GA1 (*), and *Ali. Acidocaldarius* subsp. *acidocaldarius* 104-IA (▲; at 60°C) and sterile controls with glucose (○) and sulfur (◇) at 70°C.

5.2.2.2 Dissolution of synthesized goethite via reductive bioleaching using microbial Fe(III) reduction by acidophiles under micro-aerobic conditions

When tested with mesophilic acidophiles, noticeable Fe dissolution was not observed in cell suspensions, with the exception of *Ferrimicrobium* sp. T23: The strain showed Fe dissolution, but it was only marginal (approximately 2% within 27 days). Unexpectedly, in cell suspension of the strain, Fe dissolution continuously occurred in the form of Fe(III) throughout the experiment. In general, goethite can be solubilized via reductive bioleaching using microbial Fe(III)-reducing capability, resulting in Fe dissolution with accumulation of Fe(II) in solution phase. One possible interpretation of the fact is microbial reduction of soluble Fe(III) that chemically solubilized from goethite (via a shift of chemical equilibrium of Fe(III) between in solid and soluble phase), followed by re-oxidation of Fe(II) to Fe(III) by microbial Fe(II)-oxidizing capability of the strain.

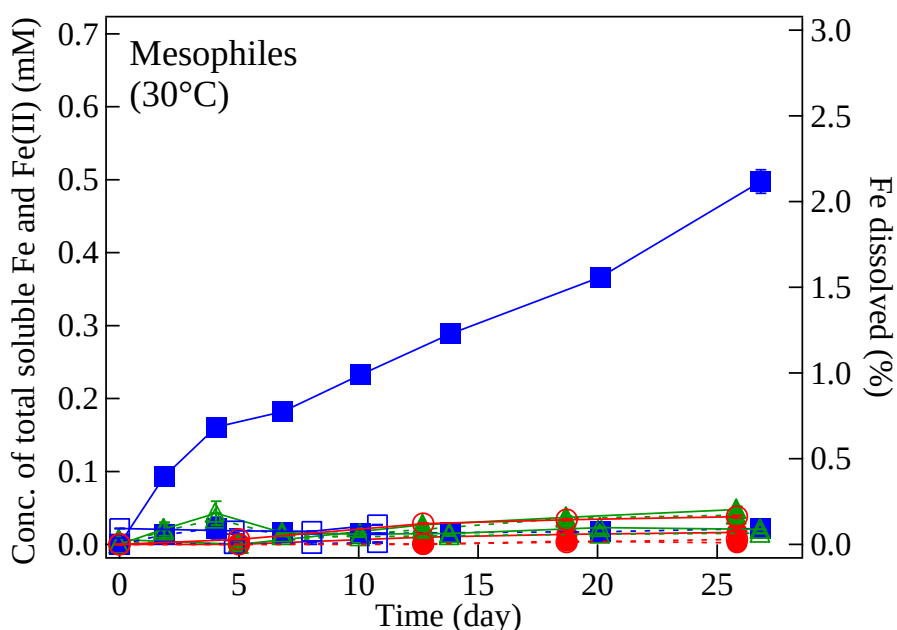


Fig. 5.5 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) in cell suspensions of *Acidiphilium* sp. SJH (●), *Ac. aromatica* PFBC (▲), and *Ferrimicrobium* sp. T23 (■), and sterile controls with glucose (○), fructose (△), and glycerol (□), respectively at 30°C.

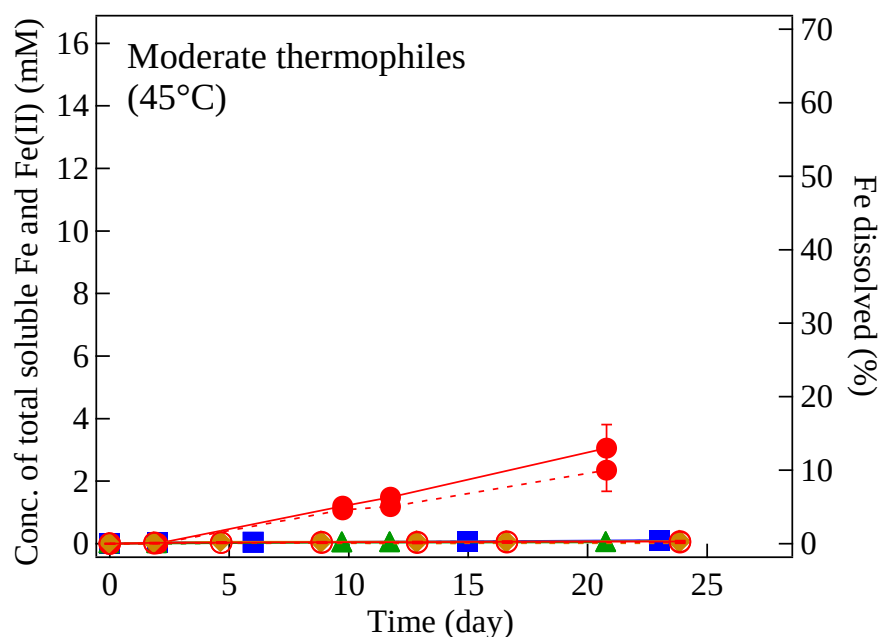


Fig. 5.6 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) in cell suspensions of *Sb. acidophilus* YTF1 (●), *Ali. acidiphilus* TA-67 (■), *Ali. acidoterrestris* GD3B (▲), and *Acidiplasma* sp. Fv-Ap (◆) and sterile controls with glucose (○), respectively at 45°C.

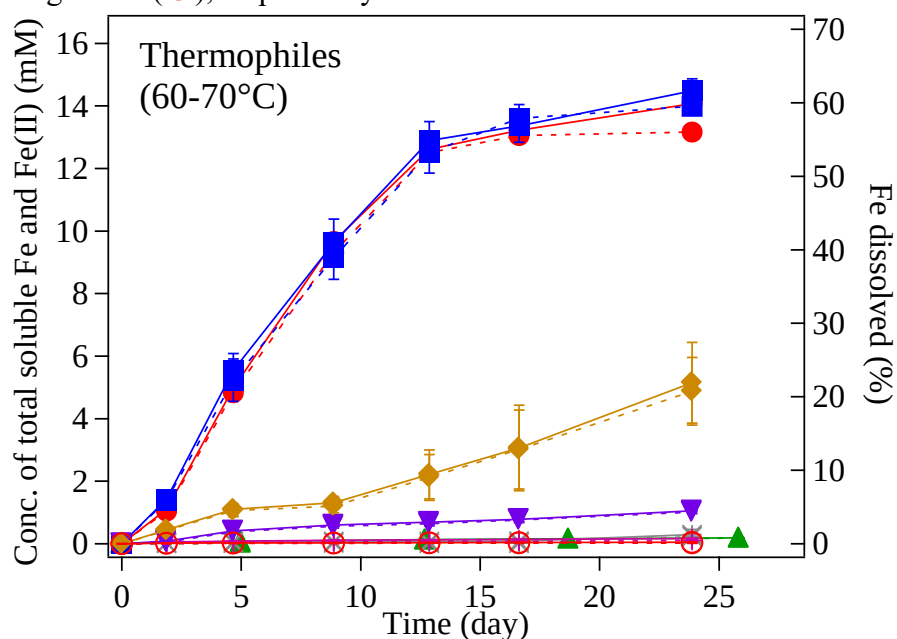


Fig. 5.7 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) in cell suspensions of *S. shibatae* B12 (●), *S. tokodaii* 7 (■), *S. metallicus* Kra23 (◆), *S. acidocaldarius* 98-3 (+), *S. solfataricus* P1 (▼), *Sulfolobus* sp. GA1 (*), and *Ali. Acidocaldarius* subsp. *acidocaldarius* 104-IA (▲; at 60°C) and sterile controls with glucose (○) and sulfur (◇), respectively at 70°C.

As for Fe dissolution from goethite by moderate thermophiles, *Sb. acidophilus* YTF1 was only the strain that solubilized goethite via reductive bioleaching. Although the strain showed the most efficient reduction capability of soluble Fe(III) among Fe(III)-reducing acidophiles tested in the previous experiment, goethite dissolution by cell suspension of the strain reached only below 15% within 21 days.

When tested with thermophiles, *S. shibatae* B12 and *S. tokodaii* 7 showed the most effective Fe dissolution capability (60% within 24 days) from goethite with accumulation of Fe(II) throughout the experiment. Although *S. acidocaldarius* 98-3 and *S. solfataricus* P1 showed reduction behaviors of soluble Fe(III) with the same degree as that of *S. shibatae* B12 in the previous experiment, the two *Sulfolobus* strains did not show remarkable Fe dissolution from goethite.

Overall, utilization of *S. shibatae* B12 and *S. tokodaii* 7 is likely the best selection for Ni extraction through reductive bioleaching of limonite in the next experiments. From an economical point of view, treatment cost in practical operation at 70°C is much higher than that at moderate temperature (e.g., at 45°C). Therefore, use of *Sb. acidophilus* YTF1 for Ni extraction through reductive bioleaching of limonite was also tested in the next sections.

5.3 Section 2 Reductive bioleaching of acid washed Indonesian limonite ore for Ni recovery using moderately thermophilic and thermophilic Fe(III)-reducing acidophiles under micro-aerobic conditions

5.3.1 Materials and methods

Cells of *S. shibatae* B12 and *S. tokodaii* 7 were pre-grown, harvested in the late exponential phase by centrifugation ($12,000 \times g$, 10 min, 40°C), washed twice with HBS medium (pH 2.0 with H_2SO_4). To evaluate microbial dissolution of limonite under micro-aerobic condition, each of the strain was re-suspended to 1×10^9 cells/mL in 50 mL of HBS medium (pH 1.4 with H_2SO_4) containing 2% (w/v) limonite, 0.01% (w/v) yeast extract, and 10 mM citrate (as a buffer solution). To establish micro-aerobic condition, 20-mL serum bottles containing the above 50 mL reaction medium were maintained at 70°C with shaking (150 rpm). Samples were regularly taken to monitor the concentrations of Fe(II), total soluble Fe, Ni, and Ca, solution redox potentials, and pH values. All the experiments were carried out in duplicates. Residues were collected by filtration at the end of the experiment, freeze-dried overnight, and then applied to XRF analysis.

Table 5.2 Mineralogical composition of raw and acid washed Indonesian limonite according to XRF analysis.

mass % Elements	Raw limonite	Acid washed limonite
Fe	38.6	40.8
Si	10.2	9.3
Mg	5.1	4.3
Al	2.8	3.2
Ni	2.8	3.0
Ca	2.0	1.1
Cr	1.5	1.6
Mn	0.43	0.46
S	0.028	0.036
Zn	0.052	0.054
V	0.026	0.026
K	0.035	< 1 $\mu\text{g/g}$
Sr	0.0047	0.0038

5.3.2 Results and discussion

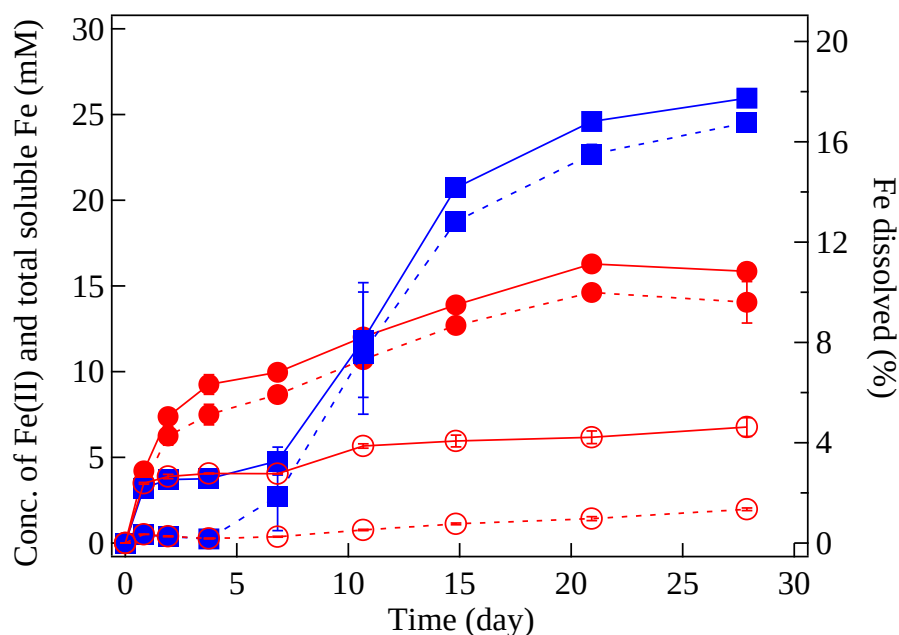


Fig. 5.8 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) in cell suspensions of *S. shibatae* B12 (●) and *S. tokodaii* 7 (■), and in sterile controls (○), respectively at 70°C.

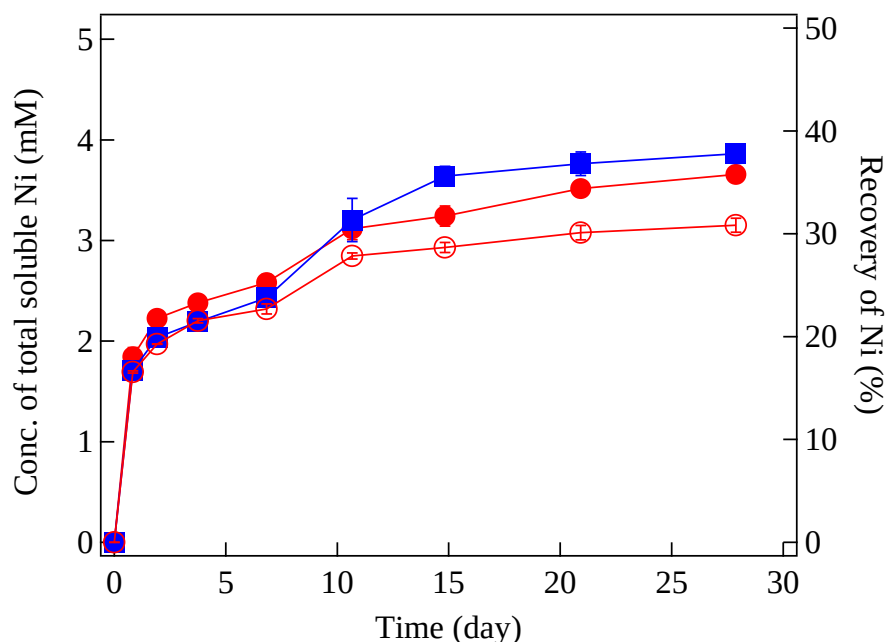


Fig. 5.9 Changes in the concentration of total soluble Ni in cell suspensions of *S. shibatae* B12 (●) and *S. tokodaii* 7 (■), and in sterile controls (○), respectively at 70°C.

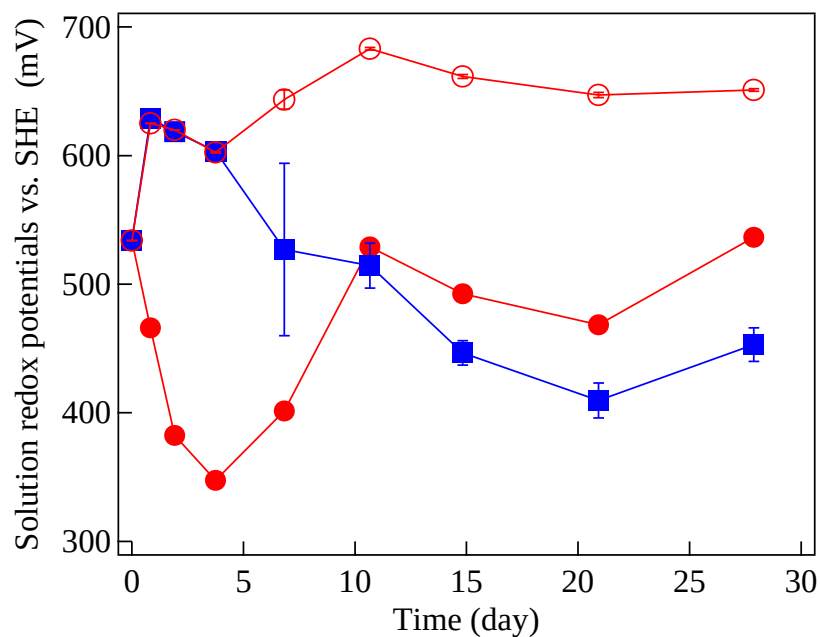


Fig. 5.10 Changes in the solution redox potentials in cell suspensions of *S. shibatae* B12 (●), *S. tokodaii* 7 (■), and in sterile controls (○), respectively at 70°C.

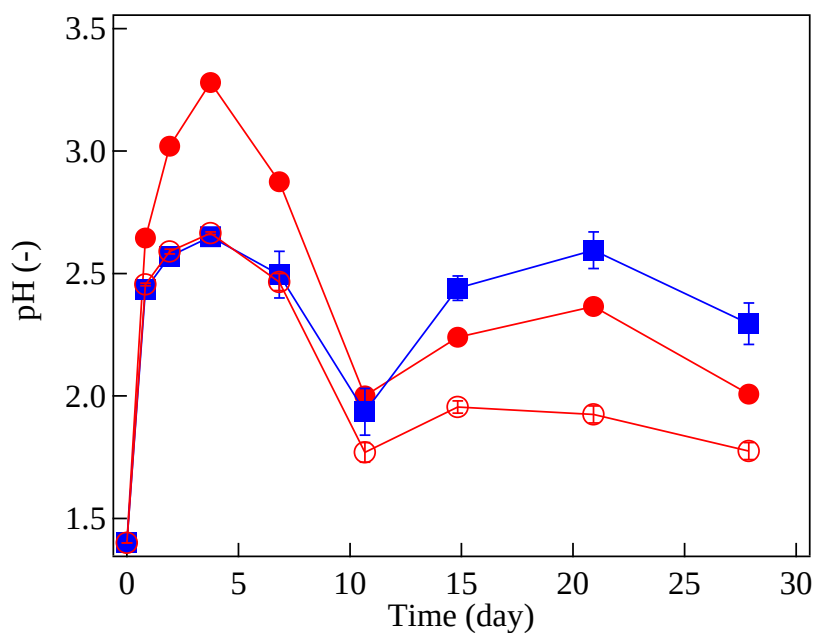


Fig. 5.11 Changes in the pH values in cell suspensions of *S. shibatae* B12 (●), *S. tokodaii* 7 (■), and in sterile controls (○), respectively at 70°C.

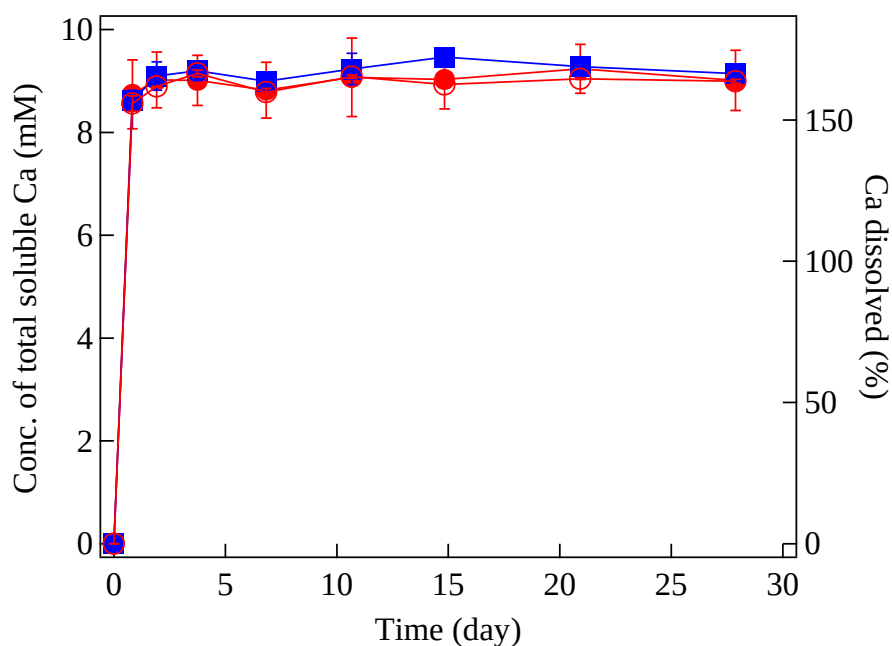


Fig. 5.12 Changes in the concentration of total soluble Ca in cell suspensions of *S. shibatae* B12 (●) and *S. tokodaii* 7 (■), and in sterile controls (○), respectively at 70°C.

Table 5.3 Mineralogical composition of acid washed limonite, residues in cell suspensions of *S. shibatae* B12 and *S. tokodaii* 7, and in sterile controls, according to XRF analysis.

Elements mass %	Before bioleaching	After bioleaching		
	Acid washed limonite	Cell suspensions of <i>S. shibatae</i> B12	Cell suspensions of <i>S. tokodaii</i> 7	Sterile controls
Fe	40.8	44.7	42.0	42.6
Si	9.3	11.2	12.9	11.9
Mg	4.3	1.3	1.8	1.9
Al	3.2	1.7	1.7	2.2
Ni	3.0	1.6	1.4	1.5
Ca	1.1	0.18	0.21	0.21
Cr	1.6	1.6	1.6	1.6
Mn	0.46	0.23	0.21	0.24
S	0.036	0.53	0.49	0.51
Zn	0.054	0.031	0.027	0.029
V	0.026	0.023	0.023	0.027
K	< 1 µg/g	0.052	0.048	0.030
P	0.0038	0.058	0.068	0.042

In the beginning of the experiment, significant dissolution of Ca was immediately confirmed, followed by rapid increase in pH values from 1.4 to 2.5 within 1 day. This might be caused by acid dissolution of Ca contents (in the form of CaO) in limonite ore, as shown in Eq. 5.3.



In order to maintain pH values below 2.0 to avoid from forming ferric precipitates, concentrated sulfuric acid was added to all the media at day 4, 7, and 21. Although sudden increase in Ni concentration was observed at day 1 in cell suspensions and sterile controls, Fe dissolution was not associated with it, indicating that origin of its Ni dissolution was not goethite (possibly from serpentine).

In cell suspensions of *S. shibatae* B12, Fe dissolution mostly in the form of Fe(II) via microbial Fe(III) reduction was observed, while chemical dissolution of Fe mostly in the form of Fe(III) was observed in cell suspensions of *S. tokodaii* 7 and sterile controls, resulting in decrease in solution redox potentials in the former cell suspensions, but increase in solution redox potentials in the latter cell suspensions and sterile controls until day 1. Later, Fe dissolution in the form of Fe(II) was observed in cell suspensions of *S. tokodaii* 7, followed by a gradual decrease in solution redox potential, while Fe dissolution in sterile controls was less effective compared with that in cell suspensions. Ni dissolution in cell suspensions and sterile controls were similarly behaved throughout the experiment, although Fe dissolution behaviors in cell suspensions are different from that in sterile controls. This indicates that Ni dissolution was not originated mainly from goethite dissolution: Ni might be solubilized mostly from serpentine, but not goethite, since Ni is often associated with serpentine as well as goethite.

Fe dissolution through microbial Fe(III) reduction in cell suspensions of *S. shibatae* B12 and *S. tokodaii* 7 reached 60% within 24 days. Therefore, further improvement in Ni recovery in cell suspensions can be expected by removing serpentine from original limonite ore. Since dissolution of serpentine through citrate chemical leaching has been reported (Tzeferis et al 1994, Valix et al 2001a, 2001b, Le et al 2006, Coto et al 2008, Mubarok et al 2011, Astuti et al 2015, 2016a, 2016b). In

the next experiment, limonite ore was washed with citric acid (2.3.1) in order to solubilize serpentine, but not goethite.

5.4 Section 3 Reductive bioleaching of citrate washed Indonesian limonite ore for Ni recovery using moderately thermophilic and thermophilic Fe(III)-reducing acidophiles under micro-aerobic conditions

5.4.1 Materials and methods

5.4.1.1 Reductive bioleaching of citrate washed Indonesian limonite ore for Ni recovery using cell suspensions of *S. shibatae* B12, *S. tokodaii* 7 and *Sb. acidophilus* YTF1 under micro-aerobic conditions

Cells of *S. shibatae* B12, *S. tokodaii* 7 and *Sb. acidophilus* YTF1 were pre-grown, harvested in the late exponential phase by centrifugation ($12,000 \times g$, 10 min, 40°C), washed twice with HBS medium (pH 2.0 with H_2SO_4). To evaluate Ni recovery from citrate washed limonite ore under micro-aerobic conditions, each of the strain was re-suspended into 50 mL of HBS medium (pH 1.2 with H_2SO_4) containing 2% (w/v) citrate washed-limonite, 0.01% (w/v) yeast extract, and 10 mM glucose with final cell density of 1×10^9 cells/mL. To establish micro-aerobic conditions, 50-mL serum bottles containing the above 50 mL reaction medium were maintained at 70°C (for the former two archaea) or 45°C (for the latter bacterium) with shaking (150 rpm). Samples were regularly taken to monitor the concentrations of Fe(II), total soluble Fe, Ni and Ca, solution redox potentials and pH values. All the experiments were carried out in duplicates. Residues were collected by filtration at the end of the experiment, freeze-dried overnight, and then applied to XRF analysis.

Table 5.4 Mineralogical composition of raw, acid washed and citrate washed Indonesian limonite according to XRF analysis.

mass % Elements	Raw limonite	Acid washed limonite	Citrate washed limonite
Fe	38.6	40.8	41.3
Si	10.2	9.3	12.3
Mg	5.1	4.3	3.0
Al	2.8	3.2	1.9
Ni	2.8	3.0	2.1
Ca	2.0	1.1	0.5
Cr	1.5	1.6	1.5
Mn	0.43	0.46	0.31
S	0.028	0.036	< 10 $\mu\text{g/g}$
Zn	0.052	0.054	0.037
V	0.026	0.026	0.022
K	0.035	< 1 $\mu\text{g/g}$	< 1 $\mu\text{g/g}$
Sr	0.0047	0.0038	< 0.1 $\mu\text{g/g}$

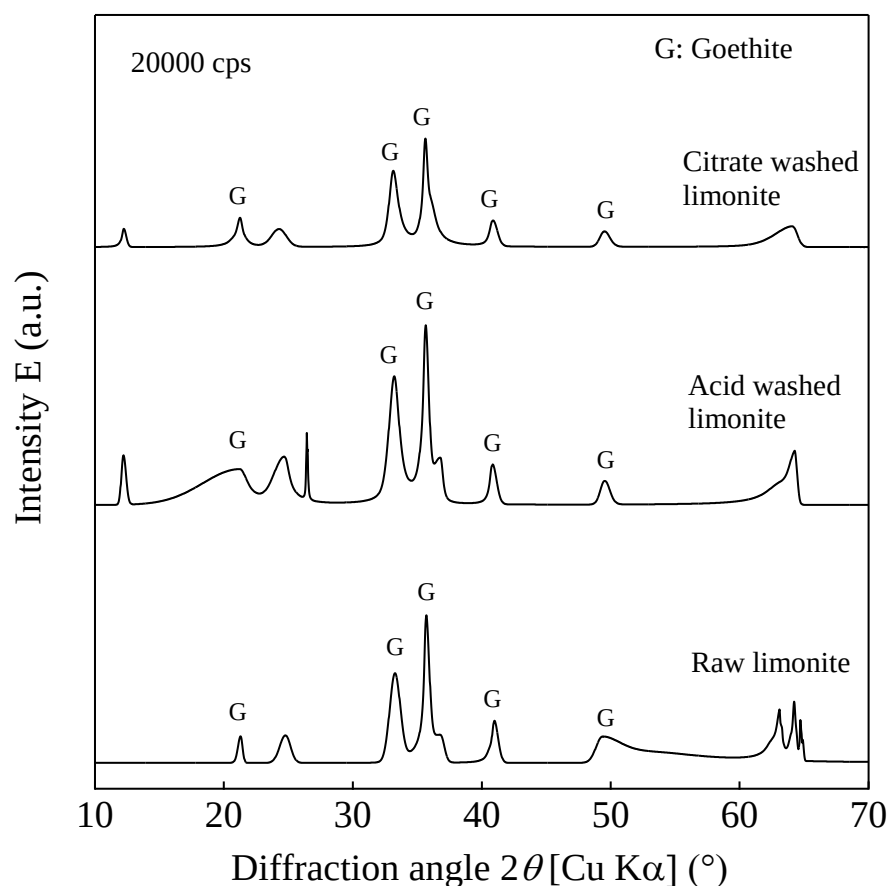


Fig. 5.13 X-ray diffraction patterns of raw, acid washed and citrate washed Indonesian limonite ore. “G” indicates peaks corresponding to goethite.

5.4.1.2 Effect of second reductive bioleaching step of bioleached residues of 1st step on Ni recovery using cell suspensions of *S. tokodaii* 7 and *Sb. acidophilus* YTF1 under micro-aerobic conditions

Residues in the previous experiment 5.4.1.1 were used to second step of reductive bioleaching. The experimental conditions in the second step was exactly same as those in the first, with the exception of the size of serum bottles (20 mL) and the media volume (20 mL) in the second step. Samples were regularly taken to monitor the concentrations of Fe(II), total soluble Fe, Ni and Ca, solution redox potentials and pH values. All the experiments were carried out in duplicates. Residues were collected by filtration at the end of the experiment, freeze-dried overnight, and then applied to XRF analysis.

5.4.1.3 Two-step reductive bioleaching of citrate washed Indonesian limonite ore for Ni recovery using *S. tokodaii* 7 and *Sb. acidophilus* YTF1 under micro-aerobic conditions

Cells of *S. tokodaii* 7 and *Sb. acidophilus* YTF1 were pre-grown, harvested in the late exponential phase by centrifugation ($12,000 \times g$, 10 min, 40°C), washed twice with HBS medium (pH 2.0 with H_2SO_4). To evaluate Ni recovery from citrate washed-limonite under micro-aerobic conditions, each of the strain was re-suspended into 50 mL of HBS medium (pH 1.2 with H_2SO_4) containing 2% (w/v) citrate washed-limonite, 0.01% (w/v) yeast extract, and 10 mM glucose with final cell density of 1×10^7 cells/mL. To establish micro-aerobic conditions, 50-mL serum bottles containing the above 50 mL reaction medium were maintained at 70°C with shaking (150 rpm).

At the end of 1st step of the experiment, leachate was removed from residues by centrifugation, and replaced with fresh HBS medium (pH 1.2 with H_2SO_4) containing 0.01% (w/v) yeast extract and 10 mM glucose was added. Note that the volume of leachate removed by centrifugation was exactly same as that of fresh HBS media. In 2nd step, fresh cells of *S. tokodaii* 7 and *Sb. acidophilus* YTF1 were not added to the media. Experimental conditions and sampling procedures in 2nd step were exactly same as those in 1st step. Samples were regularly taken to monitor the concentrations of Fe(II), total soluble Fe, Ni and Ca, solution redox potentials, pH values, and cell density. Cell density was counted (Thoma counting chamber) after centrifugation ($2,100 \times g$, 60 sec) in order to separate cells and limonite particle. All the experiments were carried out in duplicates. Residues were collected by filtration at the end of the experiment, freeze-dried overnight, and then applied to XRF analysis.

5.4.1.4 Three-step reductive bioleaching of citrate washed Indonesian limonite ore for Ni recovery using cell suspensions of *S. tokodaii* 7 and *Sb. acidophilus* YTF1 under micro-aerobic conditions

Cells of *S. tokodaii* 7 and *Sb. acidophilus* YTF1 were pre-grown, harvested in the late exponential phase by centrifugation ($12,000 \times g$, 10 min, 40°C), washed twice with HBS medium (pH 2.0 with H_2SO_4). To evaluate Ni recovery from citrate washed-limonite under micro-aerobic conditions, each of the strain was re-suspended into 50

mL of HBS medium (pH 1.2 with H₂SO₄) containing 2% (w/v) citrate washed-limonite, 0.01% (w/v) yeast extract, and 10 mM glucose with final cell densities of 1×10^9 cells/mL. To establish micro-aerobic conditions, 50-mL serum bottles containing the above 50 mL reaction medium were maintained at 70°C with shaking (150 rpm).

At the end of 1st step of the experiment, leachate was removed from residues by centrifugation, and replaced with fresh HBS medium (pH 1.2 with H₂SO₄) containing 0.01% (w/v) yeast extract and 10 mM glucose was added. Note that the volume of leachate removed by centrifugation was exactly same as that of fresh HBS media. In 2nd step, two cell suspensions were prepared; cell suspensions that fresh cells were added (to the density of fresh cells of 1×10^9 cells/mL) or not added. Experimental conditions and sampling procedures in 2nd step were exactly same as those in 1st step. As for 3rd step, the same procedure was repeated. Samples were regularly taken to monitor the concentrations of Fe(II), total soluble Fe, Ni, Ca, Mg, and Al, solution redox potentials and pH values. All the experiments were carried out in duplicates. Residues were collected by filtration at the end of the experiment, freeze-dried overnight, and then applied to XRF analysis.

5.4.1.5 “Modified” three-step reductive bioleaching of citrate washed Indonesian limonite ore for Ni recovery using inoculated cultures and cell suspensions of *S. tokodaii* 7 and *Sb. acidophilus* YTF1 under micro-aerobic conditions

In this experiment, some experimental conditions in the previous experiment (5.4.1.4) were modified as follows. Cell suspensions (1×10^9 cells/mL) and inoculated cultures (1×10^7 cells/mL) were prepared, and no additional cells were added at the latter steps. At 2nd and 3rd steps, initial pH of fresh HBS media were set to 1.35 and 1.7, respectively. Note that the volume of fresh HBS media was fixed to 50 mL at all the steps. Other experimental conditions were exactly same as those in the previous experiment (5.4.1.4).

5.4.2 Results and discussion

5.4.2.1 Reductive bioleaching of citrate washed Indonesian limonite ore for Ni recovery using cell suspensions of *S. shibatae* B12, *S. tokodaii* 7 and *Sb. acidophilus* YTF1 under micro-aerobic conditions

Rapid increase in Ca concentration was observed in both cell suspensions and sterile controls right after the addition of limonite ore to the media, followed by increase in pH values (from 1.2 to 1.4), due to acid dissolution: Approximately 60% of Ca dissolved. After an immediate Ni dissolution at the beginning of the experiment in both cell suspensions and sterile controls via acid dissolution, Ni recovery in cell suspensions was obviously improved compared with that in sterile controls: Ni recovery at day 30 reached 45 or 41% in cell suspensions of both thermophiles and moderately thermophile, respectively, while that in sterile controls reached only 26-28% regardless of the temperatures. In cell suspensions, the improvement in Ni recovery was implied to be due to significant microbial reduction of Fe(III) chemically solubilized from goethite: Fe dissolution in cell suspensions at day 30 reached 40-45%, whereas below 10% in sterile controls.

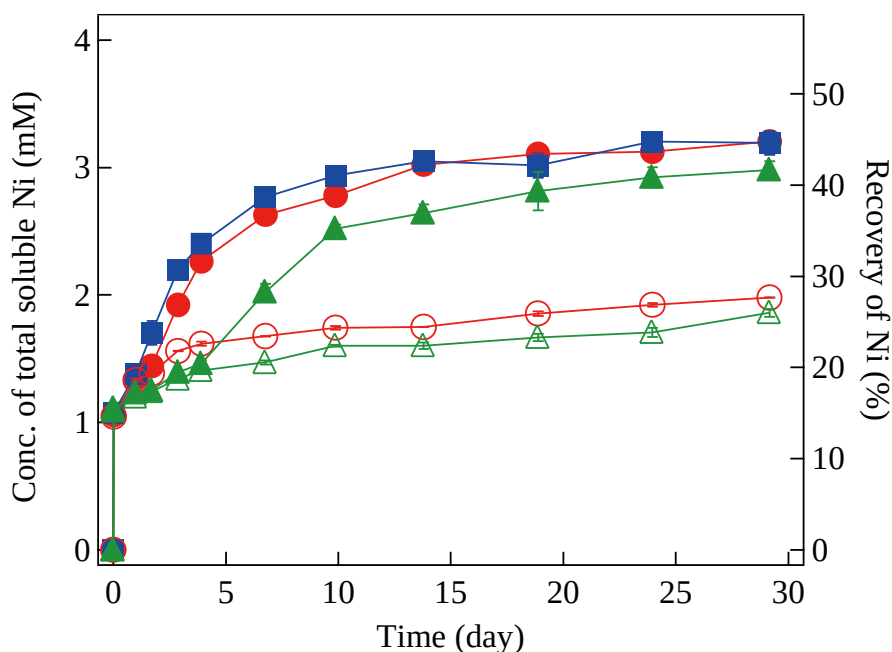


Fig. 5.14 Changes in the concentration of total soluble Ni in cell suspensions of *S. shibatae* B12 (●), *S. tokodaii* 7 (■), or *Sb. acidophilus* YTF1 (▲), and in sterile controls at 70°C (○) or 45°C (△), respectively.

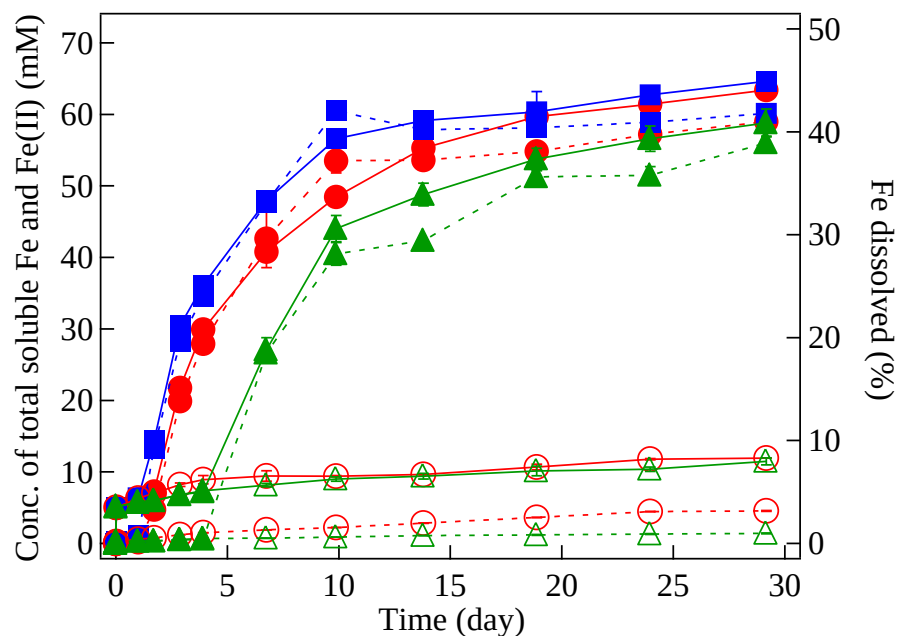


Fig. 5.15 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) in cell suspensions of *S. shibatae* B12 (●), *S. tokodaii* 7 (■), or *Sb. acidophilus* YTF1 (▲), and in sterile controls at 70°C (○) or 45°C (△), respectively.

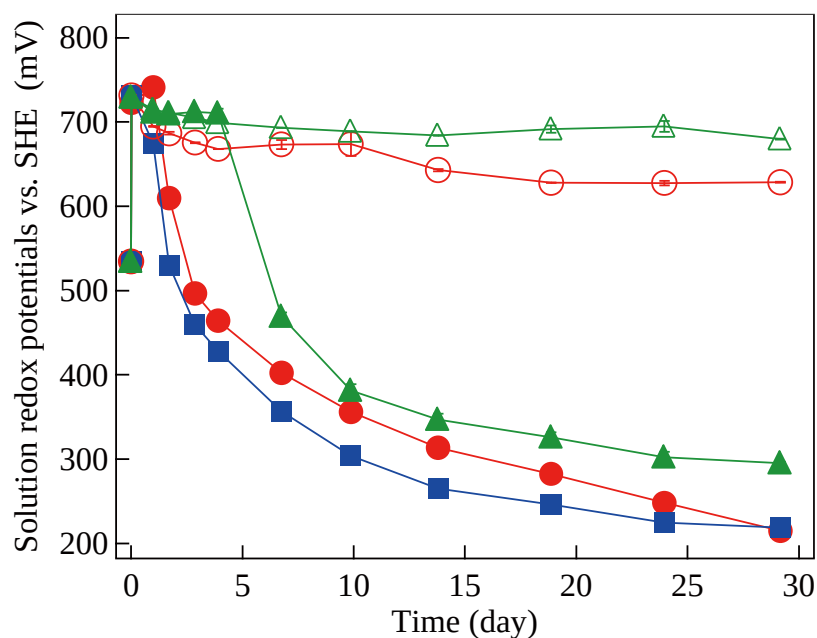


Fig. 5.16 Changes in the solution redox potentials in cell suspensions of *S. shibatae* B12 (●), *S. tokodaii* 7 (■), or *Sb. acidophilus* YTF1 (▲), and in sterile controls at 70°C (○) or 45°C (△), respectively.

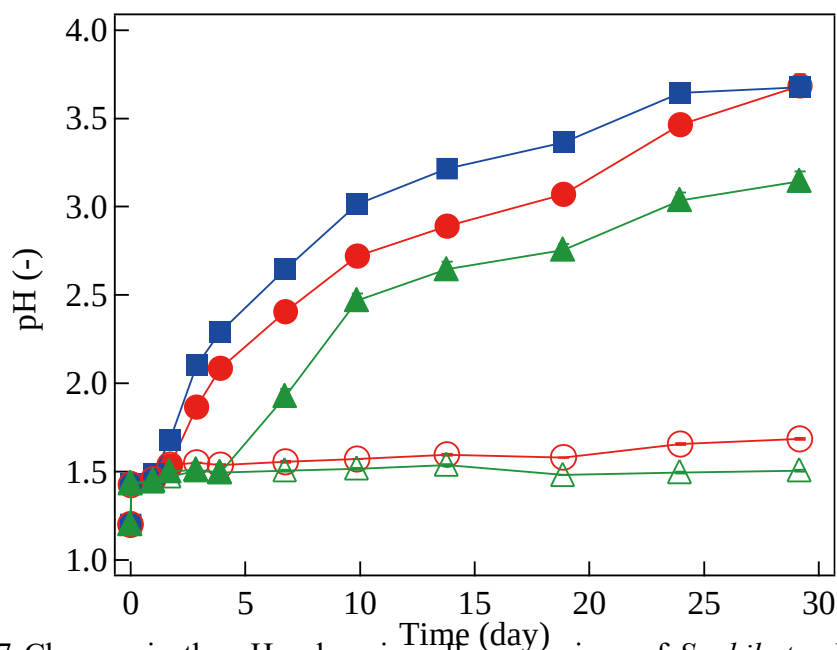


Fig. 5.17 Changes in the pH values in cell suspensions of *S. shibatae* B12 (●), *S. tokodaii* 7 (■), or *Sb. acidophilus* YTF1 (▲), and in sterile controls at 70°C (○) or 45°C (△), respectively.

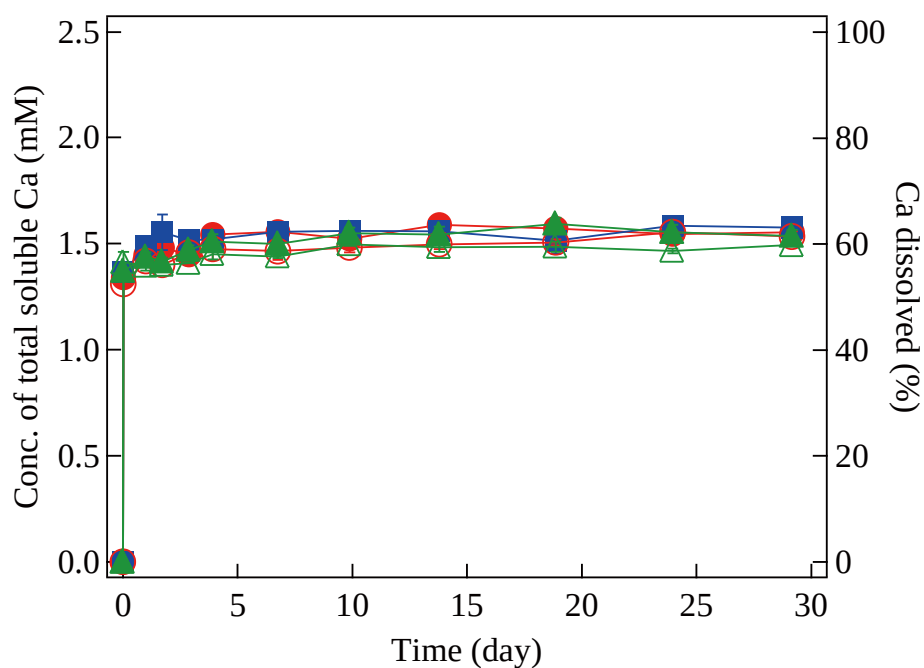


Fig. 5.18 Changes in the concentration of total soluble Ca in cell suspensions of *S. shibatae* B12 (●), *S. tokodaii* 7 (■), or *Sb. acidophilus* YTF1 (▲), and in sterile controls at 70°C (○) or 45°C (△), respectively.

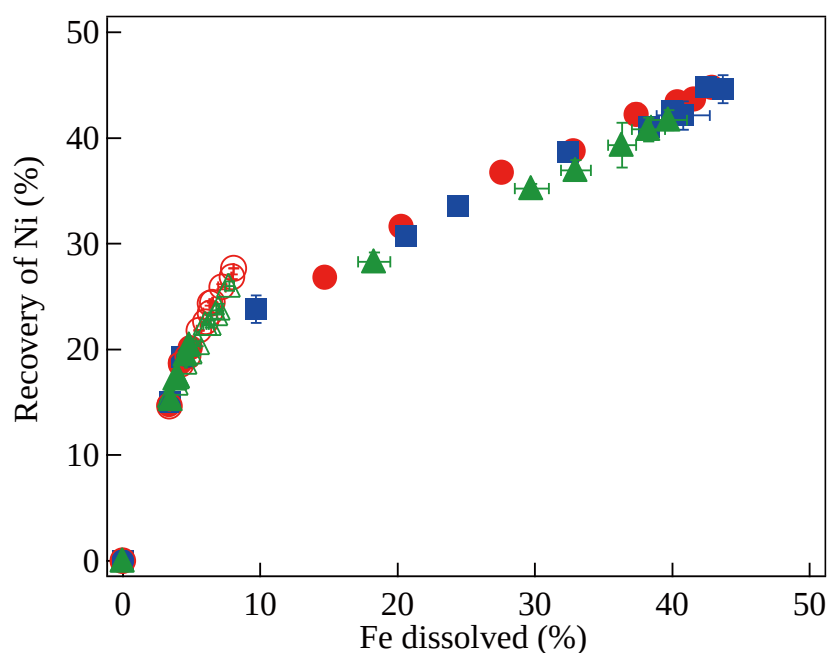


Fig. 5.19 The relationship between Ni recovery and Fe dissolution in cell suspensions of *S. shibatae* B12 (●), *S. tokodaii* 7 (■), or *Sb. acidophilus* YTF1 (▲), and in sterile controls at 70°C (○) or 45°C (△), respectively.

Table 5.5 Mineralogical composition of citrate washed limonite, residues in cell suspensions of *S. shibatae* B12, *S. tokodaii* 7, and *Sb. acidophilus* YTF1, and in sterile controls (at 70 or 45°C), according to XRF analysis.

Elements mass %	Before bioleaching	After bioleaching				
	-	70°C			45°C	
	Citrate washed limonite	Cell suspensions of <i>S. shibatae</i> B12	Cell suspensions of <i>S. tokodaii</i> 7	Sterile controls	Cell suspensions of <i>Sb. acidophilus</i> YTF1	Sterile controls
Fe	41.3	28.5	28.1	40.1	28.7	35.1
Si	12.3	21.3	21.7	14.9	20.8	17.2
Mg	3.0	2.3	2.2	1.3	2.3	1.8
Al	1.9	2.1	2.1	1.4	2.6	2.2
Ni	2.1	0.87	0.90	1.3	1.0	1.2
Ca	0.48	0.27	0.25	0.16	0.21	0.22
Cr	1.5	2.0	1.9	1.6	1.8	1.9
Mn	0.31	0.15	0.13	0.21	0.13	0.18
S	< 10 µg/g	0.37	0.37	0.58	0.44	0.44
Zn	0.037	0.027	0.022	0.023	0.022	0.025
V	0.022	0.022	0.023	0.024	0.021	0.021
K	< 1 µg/g	0.061	0.068	0.043	0.071	0.057
P	< 10 µg/g	0.086	0.10	0.015	0.082	0.043

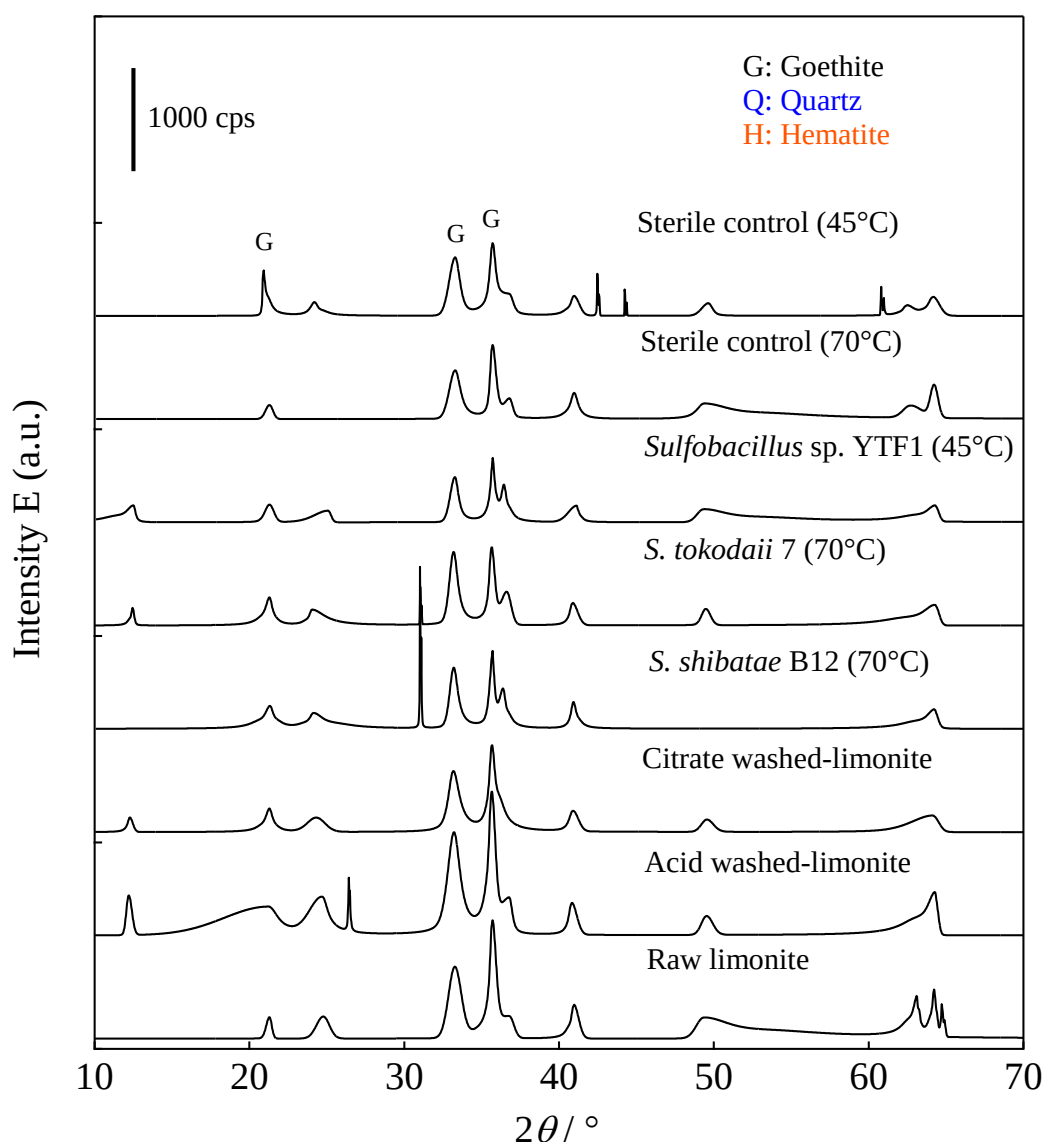


Fig. 5.20 X-ray diffraction patterns of raw, acid washed-, and citrate washed-limonite, and the residual minerals in the cell suspensions of *S. shibatae* B12, *S. tokodaii* 7, and *Sb. acidophilus* YTF1, and sterile controls at 70°C and 45°C, from the bottom to top. “G” indicates peaks corresponding to goethite.

Correlation between Ni recovery and Fe dissolution was obviously confirmed in Fig. 5.19: Ni recovery in cell suspensions was implied to be followed by Fe dissolution according to the data points in cell suspensions in the range of $10\% < \text{Fe dissolution} < 45\%$. As for their relationship in sterile controls, Ni seemed not to be extracted only via reductive bioleaching of goethite, mainly via abiotic dissolution of serpentine. According to XRF analysis, increase in Si contents and decrease in Fe and Ni contents were apparently observed in residues in cell suspensions compared with those in citrate washed limonite, indicating that goethite, but not serpentine solubilized via reductive bioleaching using microbial Fe(III) reduction.

Since solution redox potentials in cell suspensions continuously decreased throughout the experiment and reached 200-300 mV, microbial activity of Fe(III) reduction was implied to be active. However, dissolution of Fe and also Ni gradually declined after pH values increased over approximately 3.0 due to proton consumption via goethite dissolution, as shown in Eq. 5.1. The increase in pH values might cause a saturation of total soluble Fe mostly in the form of Fe(II) in the reaction media. Therefore, further Ni extraction can be expected by 2nd step of reductive bioleaching with Fe-free fresh media.

5.4.2.2 Effect of second reductive bioleaching step of bioleached residues of 1st step on Ni recovery using cell suspensions of *S. tokodaii* 7 and *Sb. acidophilus* YTF1 under micro-aerobic conditions

Second step of reductive bioleaching was subsequently carried out using bioleached residues of 1st step (in the previous experiment, 5.4.2.1). Due to significant dissolution of Ca in 1st step, its dissolution in the beginning of 2nd step was marginal, resulting in no noticeable increase in pH values right the addition of limonite, followed by its gradual increase only in cell suspensions of *S. tokodaii* 7 owing to the effective microbial Fe(III) reduction: The cell suspensions marked almost 80% as final Fe dissolution mostly as Fe(II) at day 30 (at day 60 through 1st and 2nd steps), while that in cell suspensions of *Sb. acidophilus* YTF1 reached 60% within the same period. The improvement in Fe dissolution in cell suspensions compared with that in sterile controls resulted in higher Ni recovery in the former (64 and 57% in cell suspensions of *S. tokodaii* 7 and *Sb. acidophilus* YTF1, respectively) than that in the latter (32-34%).

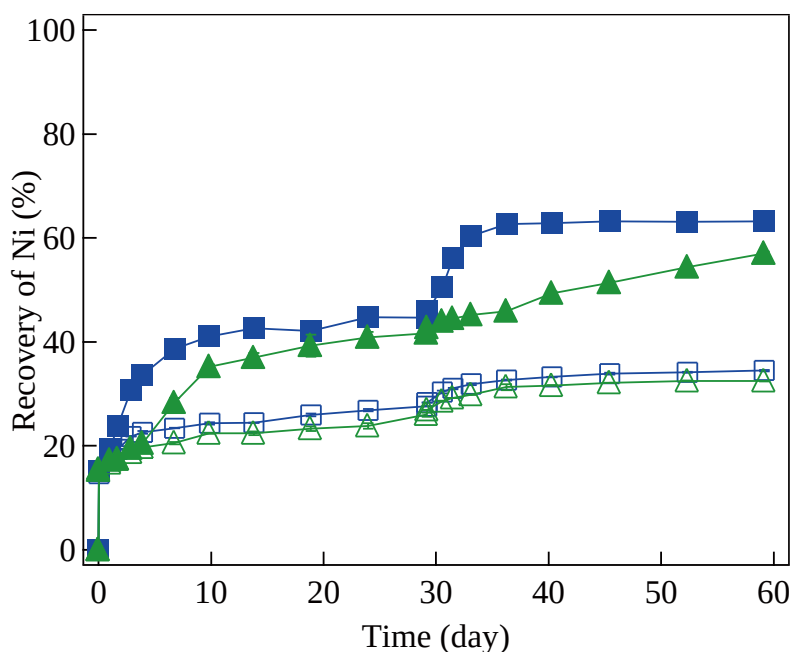


Fig. 5.21 Changes in the dissolution of total soluble Ni in cell suspensions of *S. tokodaii* 7 (■) or *Sb. acidophilus* YTF1 (▲) and in sterile controls at 70°C (□) or 45°C (△).

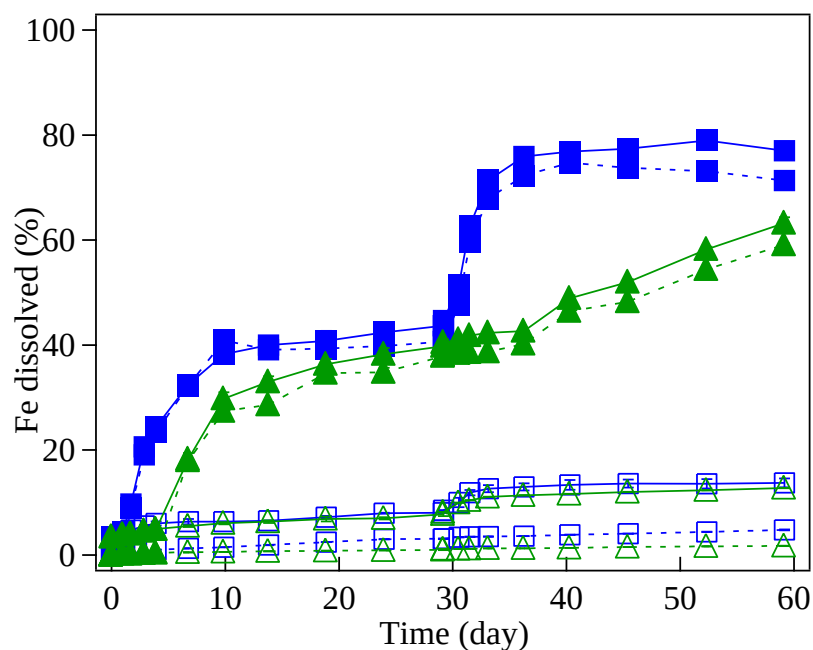


Fig. 5.22 Changes in the dissolutions of total soluble Fe (solid lines) and Fe(II) (broken lines) in cell suspensions of *S. tokodaii* 7 (■) or *Sb. acidophilus* YTF1 (▲) and in sterile controls at 70°C (□) or 45°C (△), respectively.

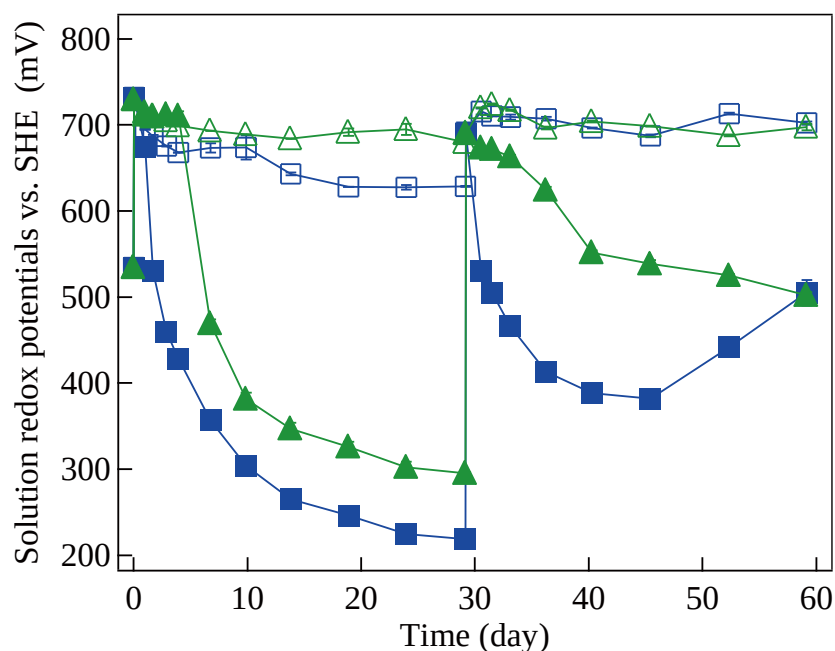


Fig. 5.23 Changes in the solution redox potentials in cell suspensions of *S. tokodaii* 7 (■) or *Sb. acidophilus* YTF1 (▲) and in sterile controls at 70°C (□) or 45°C (△).

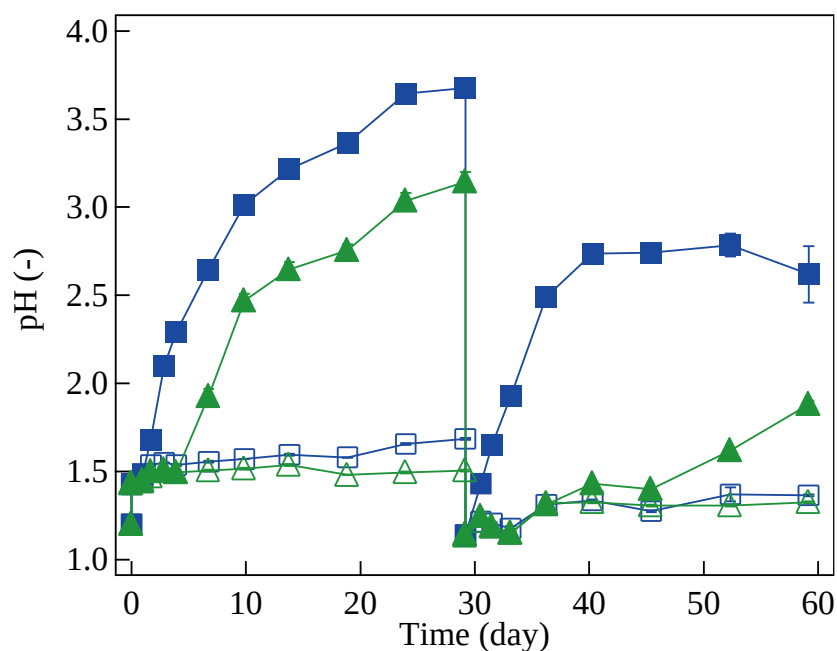


Fig. 5.24 Changes in the pH values in cell suspensions of *S. tokodaii* 7 (■) or *Sb. acidophilus* YTF1 (▲) and in sterile controls at 70°C (□) or 45°C (△).

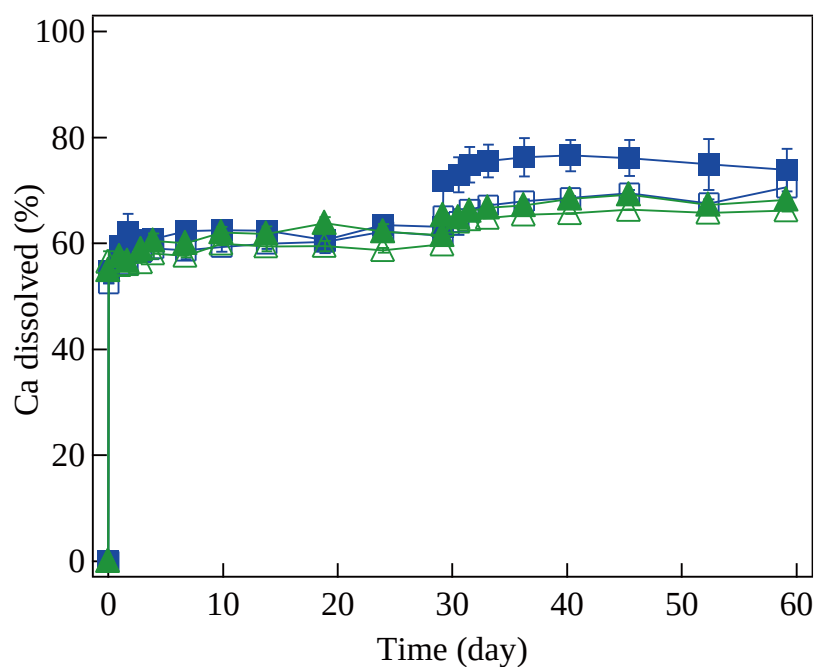


Fig. 5.25 Changes in the dissolution of total soluble Ca in cell suspensions of *S. tokodaii* 7 (■) or *Sb. acidophilus* YTF1 (▲) and in sterile controls at 70°C (□) or 45°C (△).

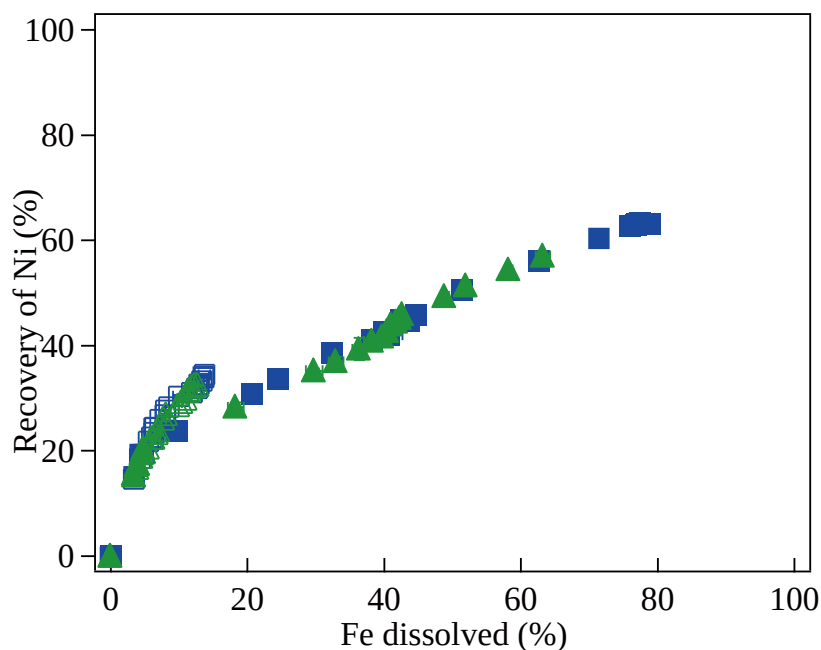


Fig. 5.26 The relationship between Ni recovery and Fe dissolution in cell suspensions of *S. tokodaii* 7 (■) or *Sb. acidophilus* YTF1 (▲) and in sterile controls at 70°C (□) or 45°C (△).

The gradual increase in pH values in both cell suspensions was caused by goethite dissolution as was mentioned previously. According to XRF analysis, further increase in Si contents and further decrease in Fe and Ni contents were observed in residues in cell suspensions in 2nd step compared with those in 1st step, indicating that goethite mainly solubilized via reductive bioleaching especially both in cell suspension.

Since the difference in Ni recoveries between cell suspensions of *S. tokodaii* 7 and *Sb. acidophilus* YTF1 were only 6% throughout the sequential reductive bioleaching test, utilization of the latter, moderately thermophilic bacterium (at 45°C), could be much applicable to practical operation for Ni extraction from an economic point of view.

Table 5.6 Mineralogical composition of citrate washed limonite, residues (2nd step) in cell suspensions of *S. tokodaii* 7 and *Sb. acidophilus* YTF1, and in sterile controls (at 70 or 45°C), according to XRF analysis.

Elements mass %	Before bioleaching	After bioleaching			
	-	70°C		45°C	
	Citrate washed limonite	Cell suspensions of <i>S. tokodaii</i> 7	Sterile controls	Cell suspensions of <i>Sb. acidophilus</i> YTF1	Sterile controls
Fe	41.3	14.2	34.4	22.8	22.3
Si	12.3	29.9	19.2	25.7	24.3
Mg	3.0	2.9	1.6	1.9	3.0
Al	1.9	2.2	0.8	1.7	2.2
Ni	2.1	0.38	0.9	0.7	0.6
Ca	0.48	0.44	0.27	0.27	0.43
Cr	1.5	2.5	1.8	1.7	3.0
Mn	0.31	< 1 µg/g	0.19	0.11	< 1 µg/g
S	< 10 µg/g	0.37	0.29	0.74	0.38
Zn	0.037	0.021	0.021	0.021	0.022
V	0.022	0.020	0.023	0.022	0.032
K	< 1 µg/g	0.125	0.018	0.073	0.048
P	< 10 µg/g	0.27	0.094	0.086	0.13
Ti	< 1 µg/g	0.18	< 1 µg/g	< 1 µg/g	< 1 µg/g

5.4.2.3 Two-step reductive bioleaching of citrate washed Indonesian limonite ore for Ni recovery using *S. tokodaii* 7 and *Sb. acidophilus* YTF1 under micro-aerobic conditions

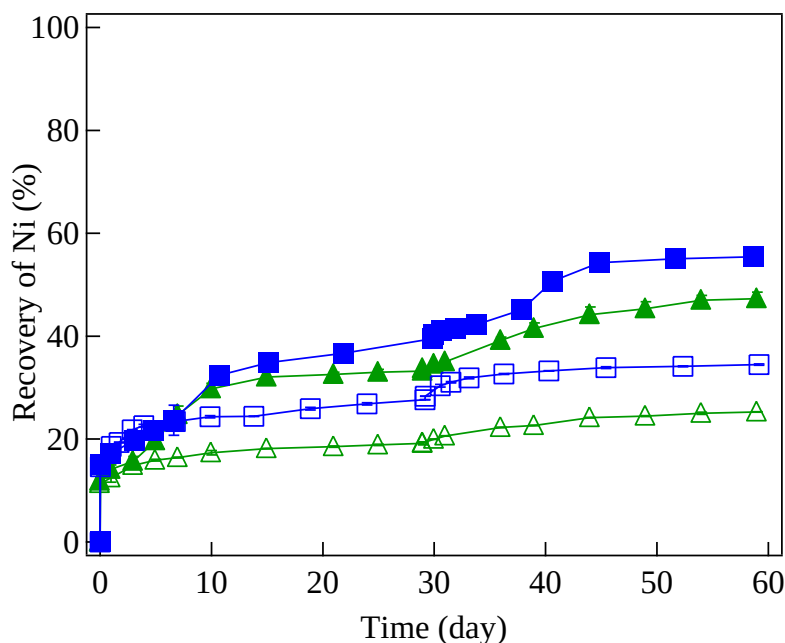


Fig. 5.27 Changes in the dissolution of total soluble Ni in inoculated cultures of *S. tokodaii* 7 (■) or *Sb. acidophilus* YTF1 (▲) and in sterile controls at 70°C (□) or 45°C (△).

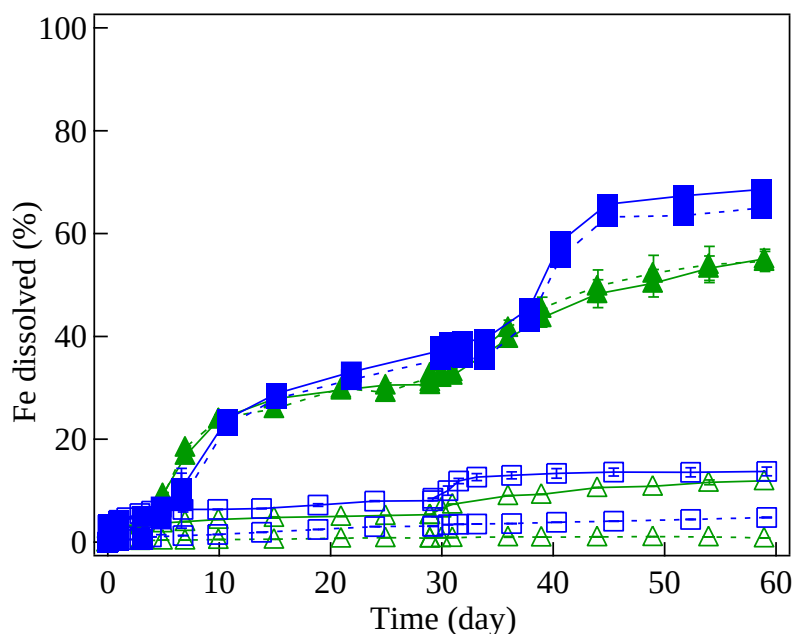


Fig. 5.28 Changes in the dissolutions of total soluble Fe (solid lines) and Fe(II) (broken lines) in inoculated cultures of *S. tokodaii* 7 (■) or *Sb. acidophilus* YTF1 (▲) and in sterile controls at 70°C (□) or 45°C (△), respectively.

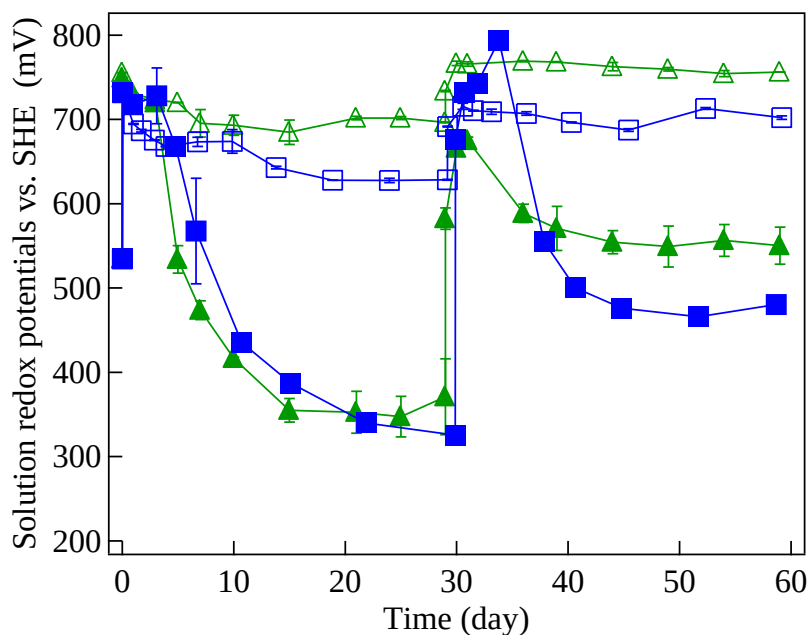


Fig. 5.29 Changes in the solution redox potentials in inoculated cultures of *S. tokodaii* 7 (■) or *Sb. acidophilus* YTF1 (▲) and in sterile controls at 70°C (□) or 45°C (△).

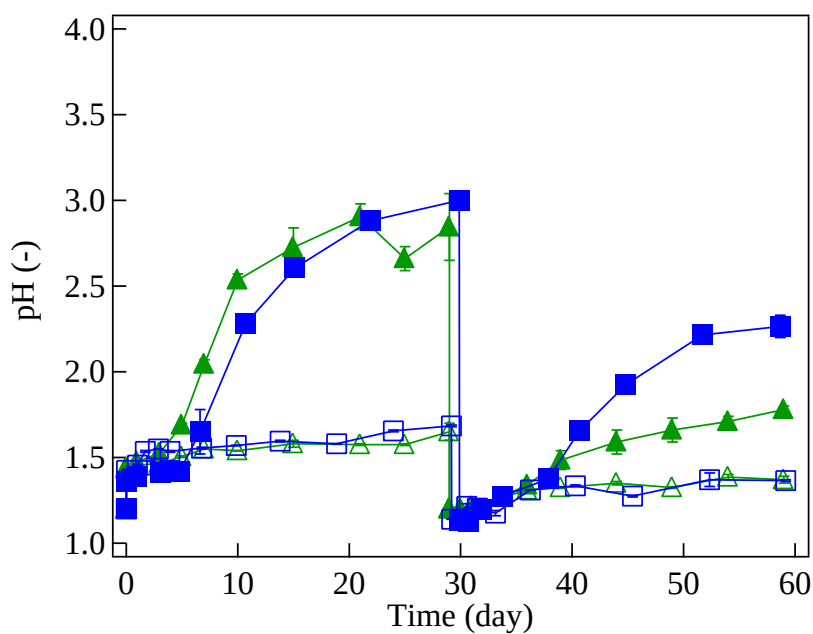


Fig. 5.30 Changes in the pH values in inoculated cultures of *S. tokodaii* 7 (■) or *Sb. acidophilus* YTF1 (▲) and in sterile controls at 70°C (□) or 45°C (△).

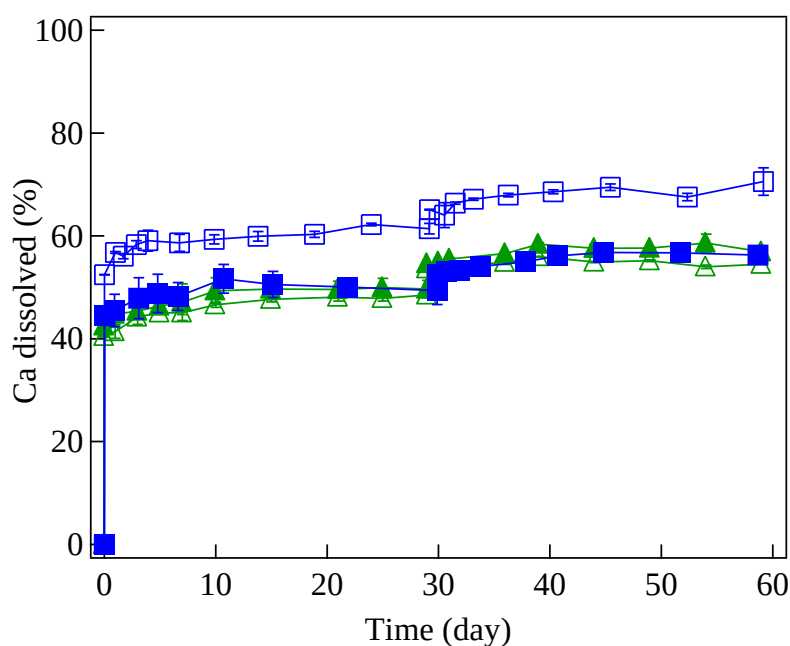


Fig. 5.31 Changes in the dissolution of total soluble Ca in inoculated cultures of *S. tokodaii* 7 (■) or *Sb. acidophilus* YTF1 (▲) and in sterile controls at 70°C (□) or 45°C (△).

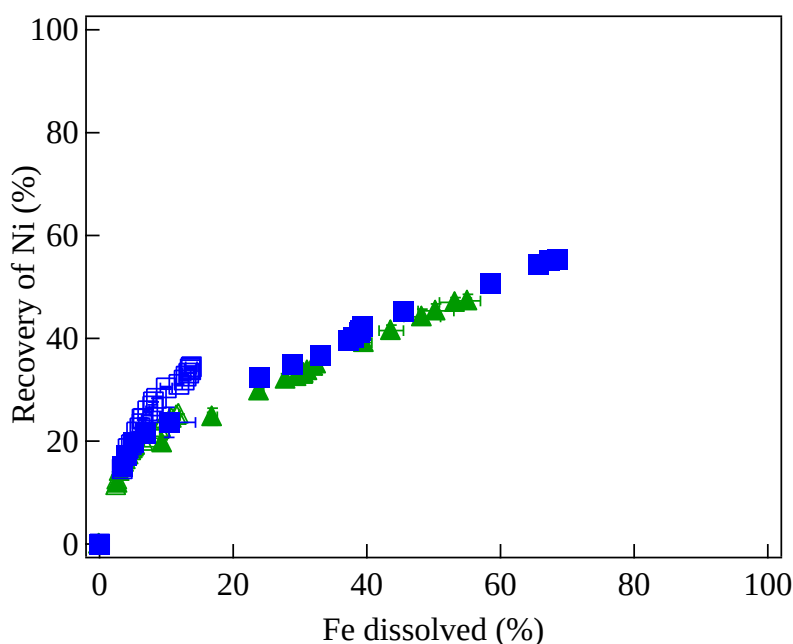


Fig. 5.32 The relationship between Ni recovery and Fe dissolution in inoculated cultures of *S. tokodaii* 7 (■) or *Sb. acidophilus* YTF1 (▲) and in sterile controls at 70°C (□) or 45°C (△).

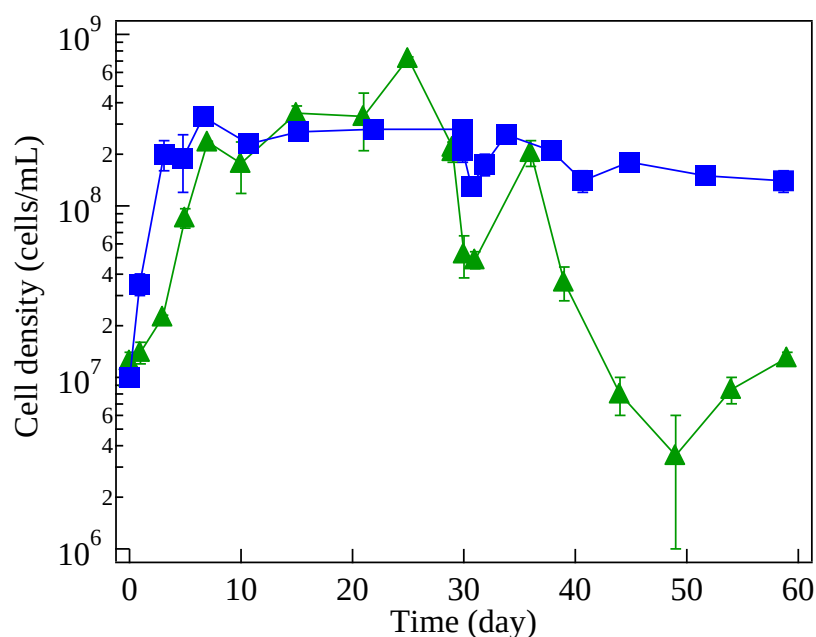


Fig. 5.33 Changes in the cell density in inoculated cultures of *S. tokodaii* 7 (■) and *Sb. acidophilus* YTF1 (▲).

Table 5.7 Mineralogical composition of citrate washed limonite, residues in inoculated cultures of *S. tokodaii* 7 and *Sb. acidophilus* YTF1, and in sterile controls, according to XRF analysis.

Elements mass %	Before bioleaching	After bioleaching			
	-	70°C		45°C	
	Citrate washed limonite	<i>S. tokodaii</i> 7	Sterile controls	<i>Sb. acidophilus</i> YTF1	Sterile controls
Fe	41.3	8.9	39.8	17.8	39.2
Si	12.3	34.4	15.5	28.5	15.0
Mg	3.0	2.3	0.8	2.3	1.3
Al	1.9	1.7	1.4	1.8	1.7
Ni	2.1	0.24	1.0	0.6	1.1
Ca	0.48	0.36	0.11	0.33	0.29
Cr	1.5	2.7	1.5	2.6	2.0
Mn	0.31	< 1 µg/g	0.21	0.23	0.22
S	< 10 µg/g	0.55	0.67	0.32	0.45
Zn	0.037	0.020	0.023	0.023	0.027
V	0.022	0.013	0.025	0.013	0.025
K	< 1 µg/g	0.066	0.045	0.055	0.012
P	< 10 µg/g	0.044	0.098	0.072	0.093
Ti	< 1 µg/g	0.17	< 1 µg/g	0.18	< 1 µg/g
Cu	< 1 µg/g	0.013	< 1 µg/g	< 1 µg/g	< 1 µg/g
As	< 0.1 µg/g	0.0027	< 0.1 µg/g	< 0.1 µg/g	< 0.1 µg/g

Dissolution of goethite phase in limonite ore and the following Ni extraction similarly behaved to those using cell suspensions in the previous experiment. Linear correlation between Ni recovery (%) and Fe dissolved (%) was confirmed right after an immediate Ni extraction at the beginning of the 1st step, as well as the previous experiment using cell suspensions. In this experiment, initial cell density was set to 1×10^7 cells/mL, resulted in a delay of decrease in redox potentials in inoculated cultures, compared with the experiment using cell suspensions. This was caused by a lag phase of activation of microbial Fe(III) reduction by the both strains. Given that Ni extraction from limonite ore corresponds to dissolution of Fe, and that the dissolution is indirectly promoted by Fe(III) reduction, less active Fe(III)-reducing capability in this experiment (compared with that using cell suspensions) resulted in lower Ni extraction (*S. tokodaii* 7, 55% with inoculated cultures, and 64% with cell suspensions; *Sb. acidophilus* YTF1, 47% with inoculated cultures, and 57% with cell suspensions, by 60 days). From an economic viewpoint, growth coupled-reductive bioleaching operation takes much more advantages over reductive bioleaching using cell suspension at the beginning of the treatment. This fact and the difference in Ni extraction (10 points in recovery percentage) between that using inoculated cultures and cell suspensions may lead to an understanding that utilization of cell suspensions is not necessarily required to economically extract Ni from limonite ores.

5.5.2.4 Three-step reductive bioleaching of citrate washed Indonesian limonite ore for Ni recovery using cell suspensions of *S. tokodaii* 7 and *Sb. acidophilus* YTF1 under micro-aerobic conditions

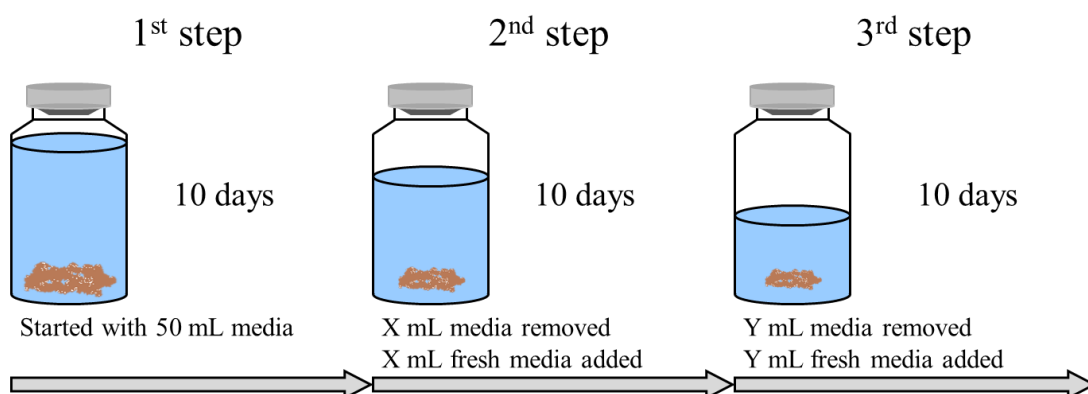


Fig. 5.34 Scheme of the experimental procedure of “three-step”.

In both cell suspensions, addition of fresh cells at each step resulted in improvement in Ni recovery to some extent for *S. tokodaii* 7, but not significant. For *Sb. acidophilus* YTF1, no enhancement in Ni recovery by the addition of fresh cells at each step was confirmed. The fact indicates that initial cell density of 1×10^9 cells/mL was enough for enhancement in Fe(III) reduction, and cells were successfully taken over to next steps. In later discussion, cell suspensions that fresh cells were not added are focused on.

Recoveries of Ni in cell suspensions of *S. tokodaii* 7 or *Sb. acidophilus* YTF1, and sterile controls at 70 or 45°C, reached 51 or 42, and 32 or 28%, respectively. Although Ni solubilized at 2nd and 3rd steps, their extraction rates were much lower than that in 1st step: In cells suspensions of *S. tokodaii* 7, Ni recoveries in 1st, 2nd, and 3rd steps were 38, 8, and 5%, respectively. In cells suspensions of *Sb. acidophilus* YTF1, Ni recoveries in s were 31, 5, and 6%, respectively. The declining in Ni recovery and also Fe dissolution might be caused by solution redox potentials. The gradual increase in final solution redox potentials at the end of each step was observed in both cell suspensions:

In cell suspensions of *S. tokodaii* 7, solution redox potentials decreased to around 300, 500, and 530 mV in the end of 1st, 2nd, and 3rd steps, respectively. The reason for the fact may be open head space in serum bottles in 2nd and 3rd steps: Volume of reaction media decreased due to approximately 1 mL of liquid sampling. In 2nd and 3rd steps, the volumes of fresh HBS media added were exactly same as those of leachates removed at the end of the previous step by centrifugation, resulting in gradual increase in volume of open head space in serum bottles. Microbial Fe(III) reduction by *S. tokodaii* 7 was found to be much more effective under anaerobic than that under micro-aerobic conditions (chapter 4), indicating that increase in dissolved oxygen levels inhibits its reduction capability of Fe(III) to Fe(II). Therefore, latter steps in this experiment was implied to become more aerobic than the former.

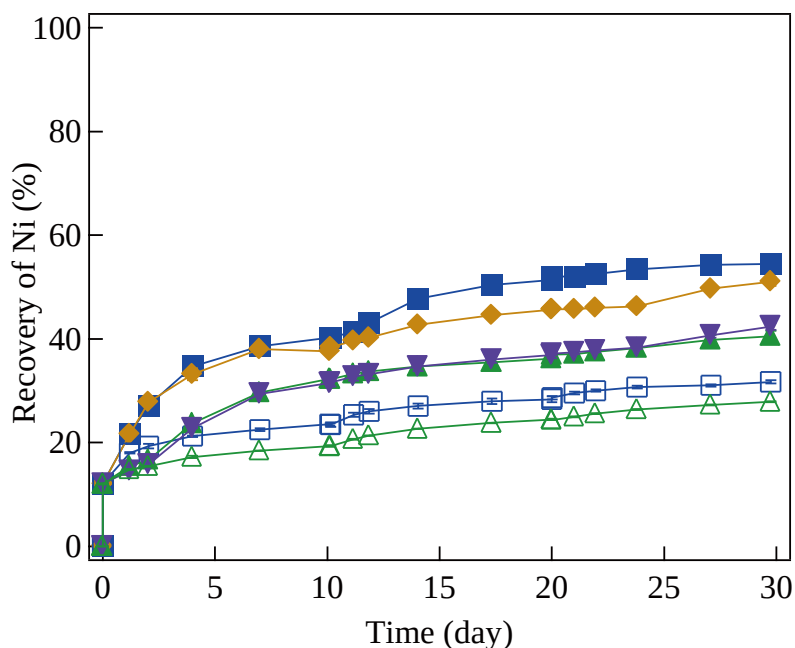


Fig. 5.35 Changes in the dissolution of total soluble Ni in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

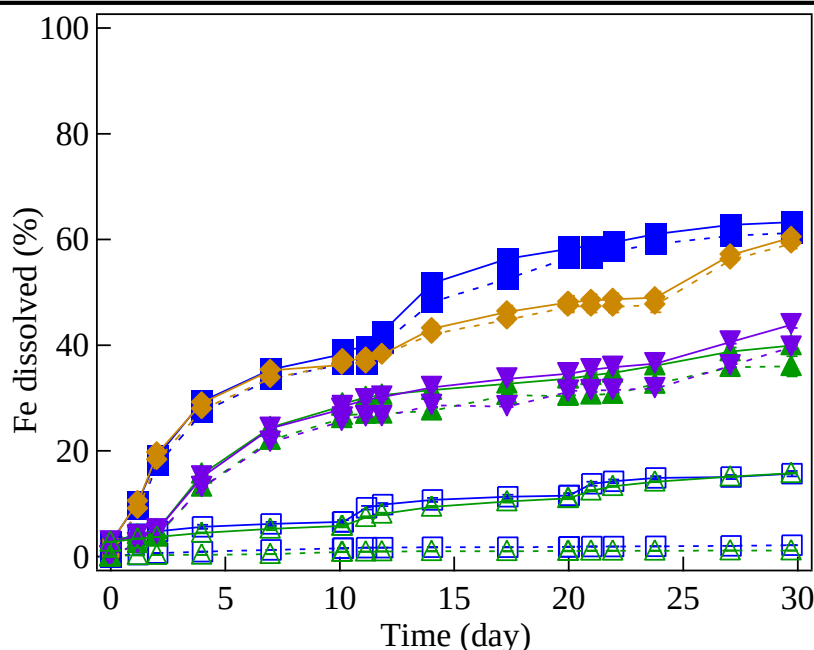


Fig. 5.36 Changes in the dissolutions of total soluble Fe (solid lines) and Fe(II) (broken lines) in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

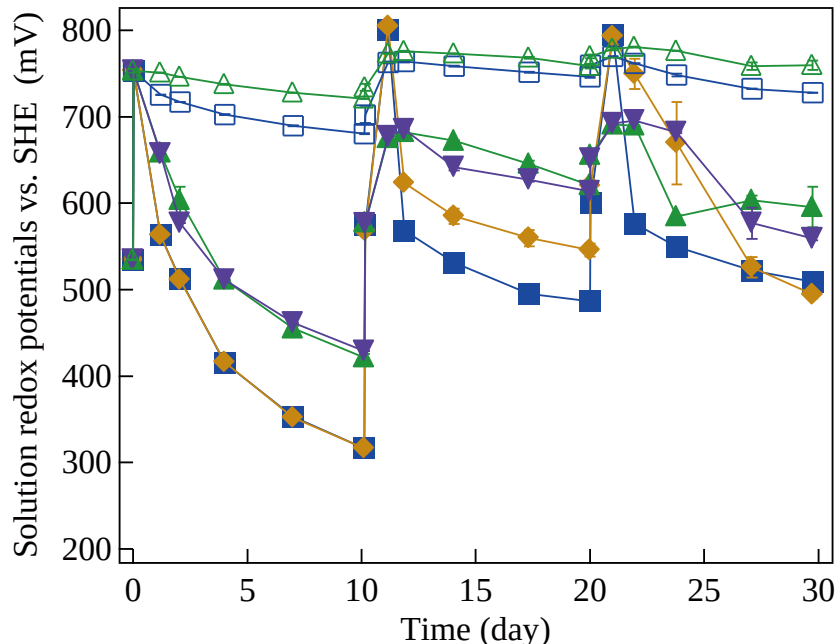


Fig. 5.37 Changes in the solution redox potentials in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

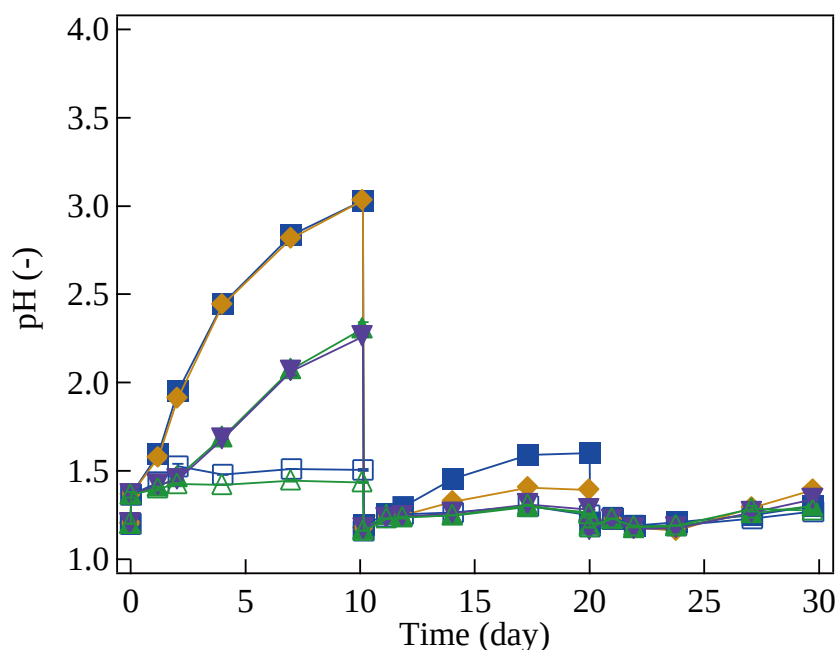


Fig. 5.38 Changes in the pH values in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

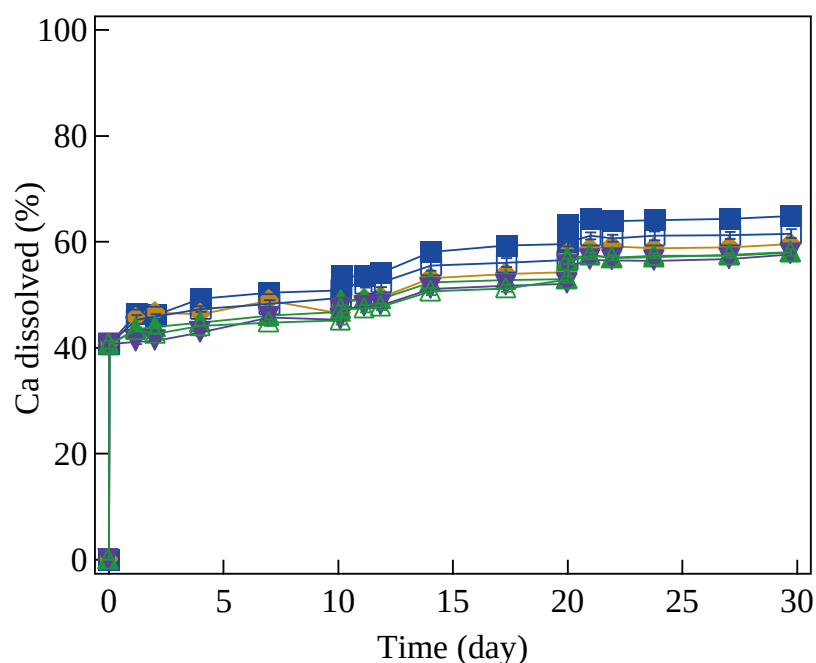


Fig. 5.39 Changes in the dissolution of total soluble Ca in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

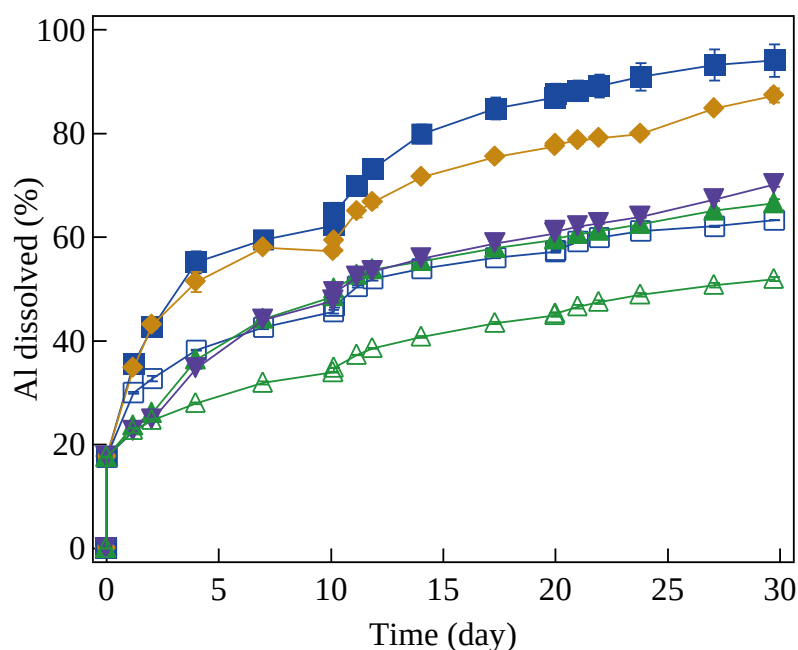


Fig. 5.40 Changes in the dissolution of total soluble Al in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

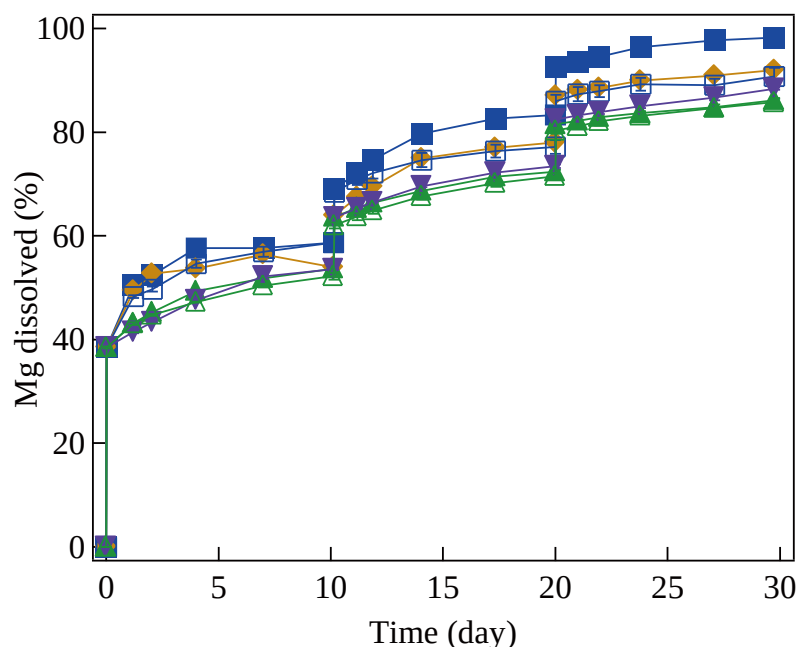


Fig. 5.41 Changes in the dissolution of total soluble Mg in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

The other possible cause for the declining in dissolution of Ni and Fe in cell suspensions, is low pH levels at 2nd and 3rd steps. Since Ca dissolution at the beginning of the last two steps was only marginal, no noticeable increase in pH values was observed right after the addition of limonite. Since optimal pH for the two Fe(III)-reducing acidophiles is 2.5-3.0, extremely acidic pHs might be implied to suppress their microbial Fe(III) reduction, resulting in less effective Fe dissolution in cell suspensions at the last two steps, especially at 3rd step.

In this experiment, concentrations of Mg and Al were monitored. Immediate dissolution of both Mg and Al was observed at the beginning of 1st step (about 40 and 20% for Mg and Al dissolution, respectively). Relationships between dissolutions of Ni and Fe, Al and Fe, Mg and Fe, Ni and Al, Ni and Mg, or Mg and Al are described in Fig. 5.42, 5.43, 5.44, 5.45, 5.46 or 5.47, respectively. In the Indonesian limonite ore used in this study, Ni was expected to associate mainly with goethite phase, which was supported by the liner correlation between dissolutions of Fe and Ni throughout the sequential steps of reductive bioleaching. Since liner relationship between dissolutions of Ni and Al was also confirmed throughout the experiment, both Al, as well as Ni, were implied to associate with goethite phase. Therefore, correlation between dissolutions of Ni and Al was successfully observed. In cell suspensions, dissolutions of Fe and Ni was observed in a molar ratio of 1:0.68, according to Fig. 5.48, indicating that Ni associates with 68% of goethite in the Indonesian limonite ore. As for Al dissolution in cell suspensions, dissolutions of Fe and Al was observed in a molar ratio of 1:1.22, according to Fig. 5.49, indicating that 1.22 moles Al associates with 1 mole of goethite. However, relationship between dissolutions of Fe and Mg was not confirmed, indicating that Mg was implied not to associate with goethite, possibly with serpentine. Therefore, it is reasonable that dissolution behavior of Mg was found not to be correlated to that of Ni and Al.

According to XRF analysis, increase in Si contents and decrease in contents of Fe, Ni, Al and Mg, were observed in residues compared with those in citrate washed limonite, as was also found in the previous experiments, indicating that goethite mainly solubilized via reductive bioleaching especially both in cell suspension.

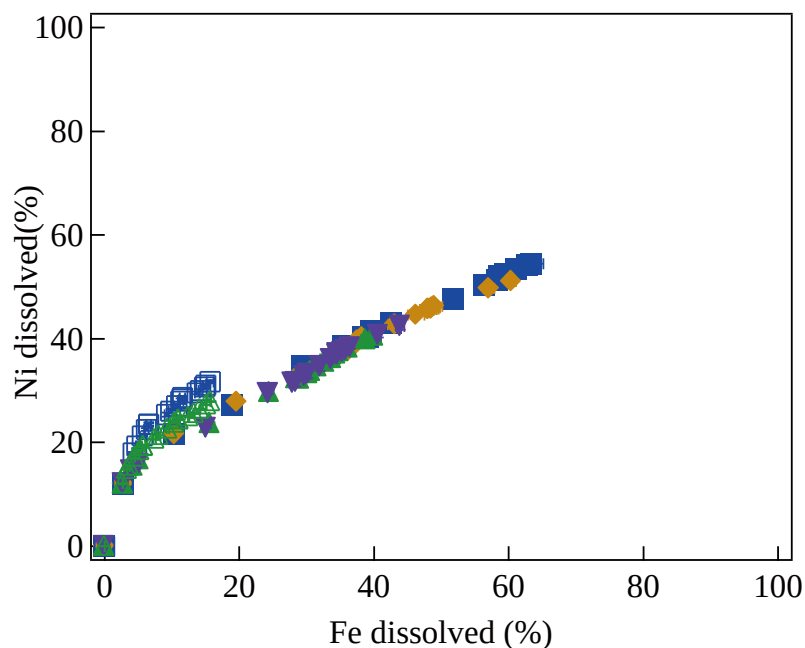


Fig. 5.42 The relationship between Ni dissolution and Fe dissolution in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

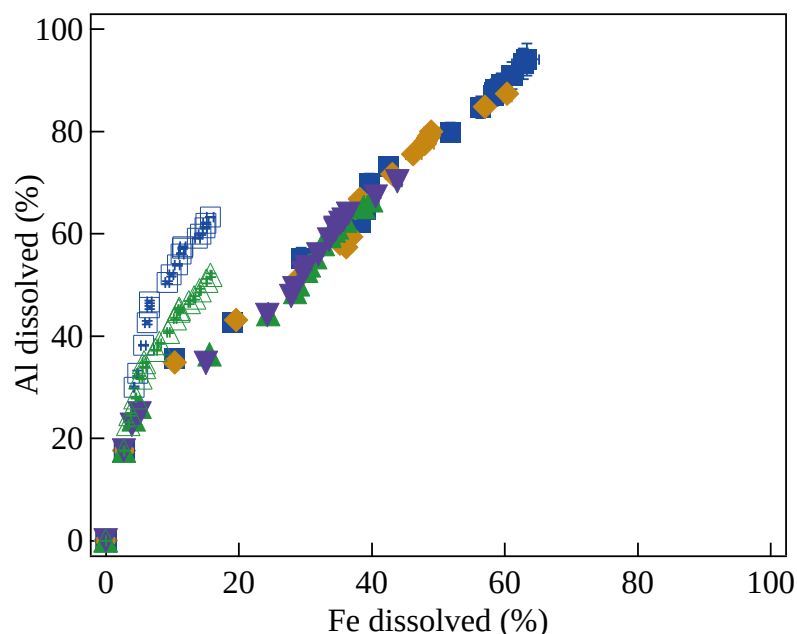


Fig. 5.43 The relationship between Al dissolution and Fe dissolution in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

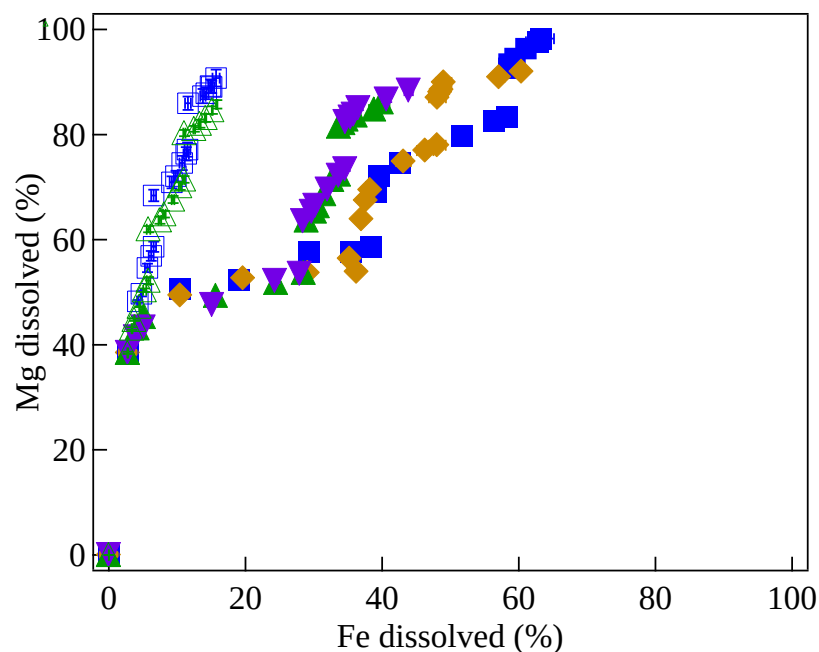


Fig. 5.44 The relationship between Mg dissolution and Fe dissolution in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

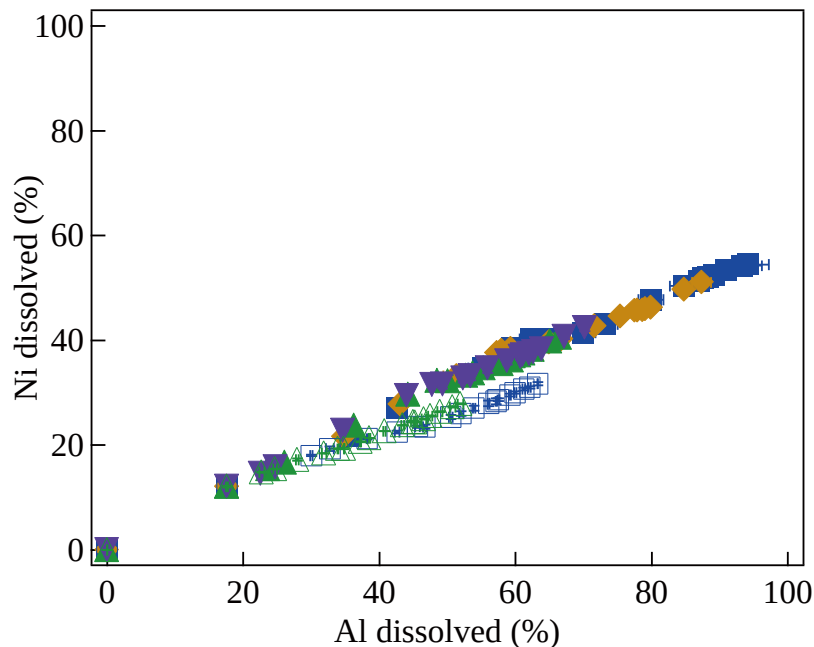


Fig. 5.45 The relationship between Ni dissolution and Al dissolution in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

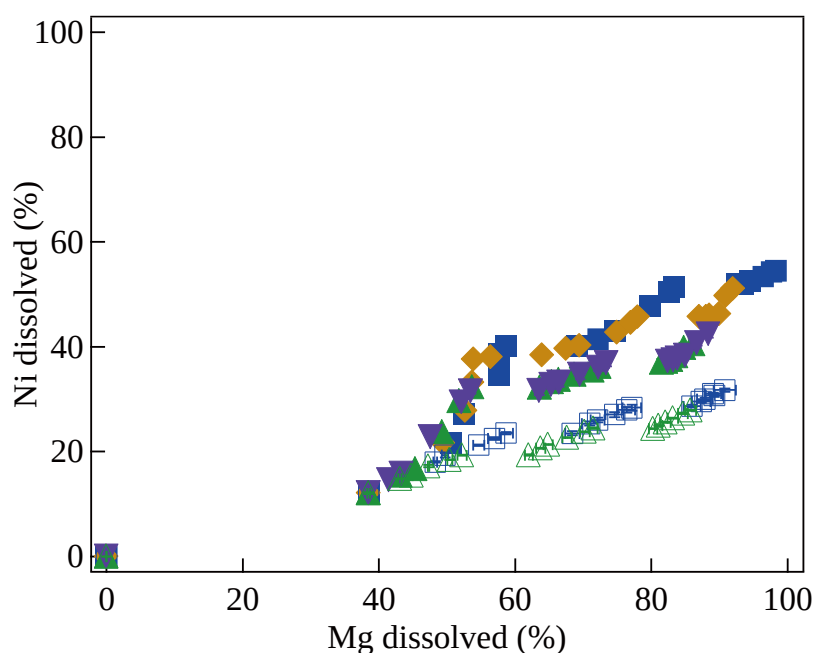


Fig. 5.46 The relationship between Ni dissolution and Mg dissolution in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

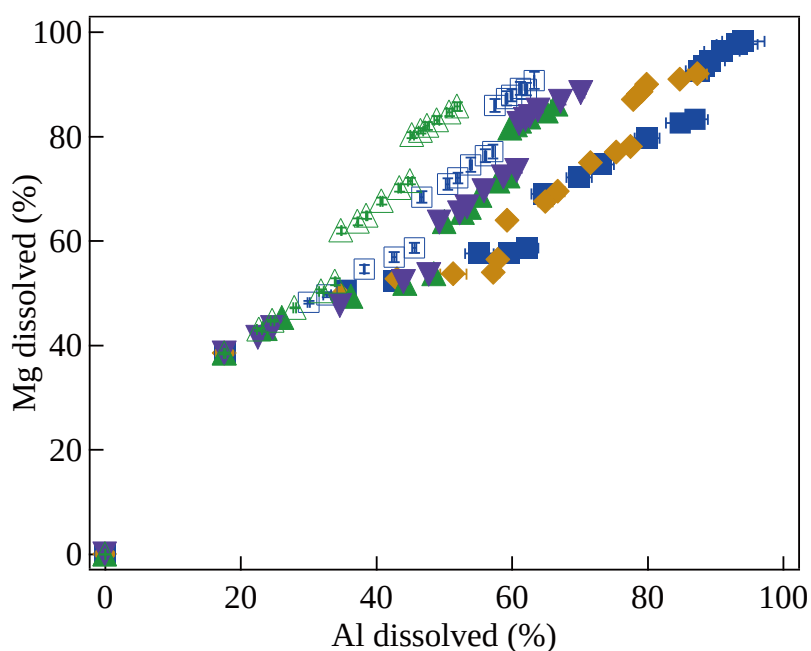


Fig. 5.47 The relationship between Mg dissolution and Al dissolution in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

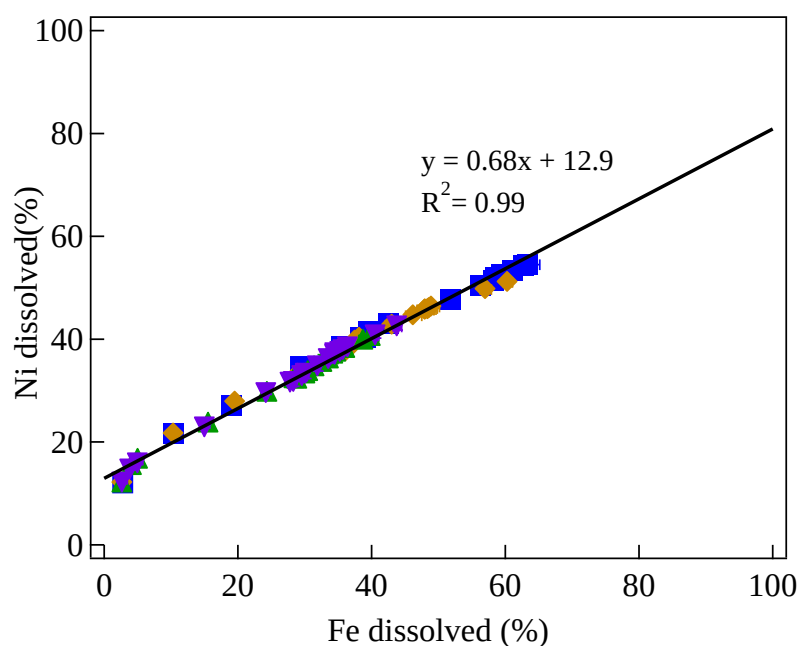


Fig. 5.48 Liner relationship between Ni dissolution and Fe dissolution in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

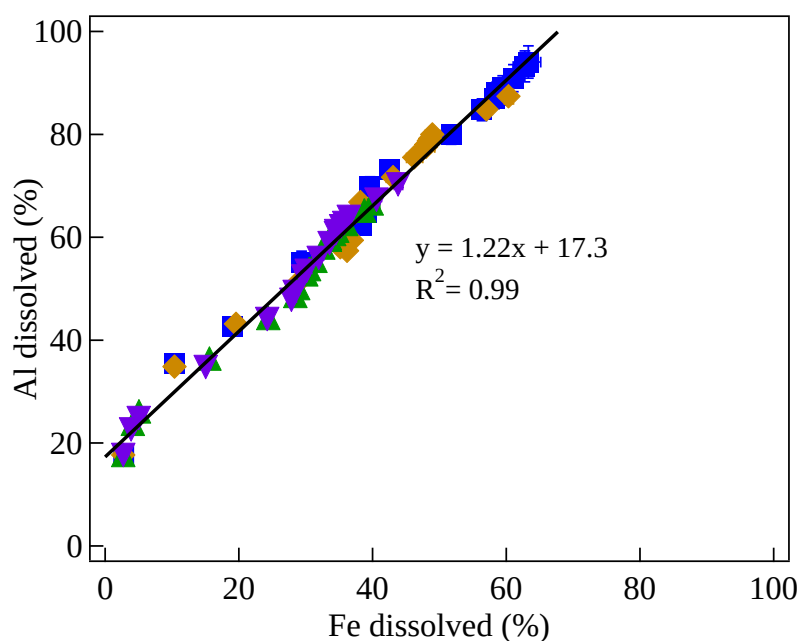


Fig. 5.49 Liner relationship between Al dissolution and Fe dissolution in cell suspensions of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼). A (■ and ▲) or B (◆ and ▼) indicate that cell suspensions that fresh cells were added or not added at each step.

Table 5.8 Mineralogical composition of citrate washed limonite, residues in cell suspensions of *S. tokodaii* 7 and *Sb. acidophilus* YTF1, and in sterile controls (at 70 or 45°C), according to XRF analysis. (A) and (B) indicate cell suspensions that fresh cells were added or not added at each step.

Elements mass %	Before bioleaching	After bioleaching					
	-	70°C			45°C		
	Citrate washed limonite	Cell suspensions of <i>S. tokodaii</i> 7 (A)	Cell suspensions of <i>S. tokodaii</i> 7 (B)	Sterile controls	Cell suspensions of <i>Sb. acidophilus</i> YTF1 (A)	Cell suspensions of <i>Sb. acidophilus</i> YTF1 (B)	Sterile controls
Fe	41.3	4.4	5.3	39.6	29.8	27.4	39.6
Si	12.3	39.1	38.7	15.2	21.1	22.8	15.4
Mg	3.0	1.4	1.1	0.89	1.4	1.4	1.1
Al	1.9	1.3	1.3	1.5	1.8	1.9	1.3
Ni	2.1	0.11	0.13	1.1	0.92	0.83	1.2
Ca	0.48	0.28	0.19	0.13	0.15	0.17	0.14
Cr	1.5	1.7	1.6	1.6	1.6	1.7	1.4
Mn	0.31	< 1 µg/g	< 1 µg/g	0.20	0.14	0.12	0.20
S	< 10 µg/g	0.77	0.87	0.75	0.93	0.77	0.71
Zn	0.037	0.014	0.014	0.025	0.022	0.022	0.024
V	0.022	0.011	0.010	0.027	0.020	0.024	0.023
K	< 1 µg/g	0.11	0.12	0.042	0.062	0.076	0.045
P	< 10 µg/g	< 10 µg/g	< 10 µg/g	0.16	0.10	0.052	0.088
Ti	< 1 µg/g	0.15	0.17	< 1 µg/g	< 1 µg/g	< 1 µg/g	< 1 µg/g
Cu	< 1 µg/g	< 1 µg/g	0.011	< 1 µg/g	< 1 µg/g	< 1 µg/g	< 1 µg/g

5.4.2.5 “Modified” three-step reductive bioleaching of citrate washed Indonesian limonite ore for Ni recovery using inoculated cultures and cell suspensions of *S. tokodaii* 7 and *Sb. acidophilus* YTF1 under micro-aerobic conditions

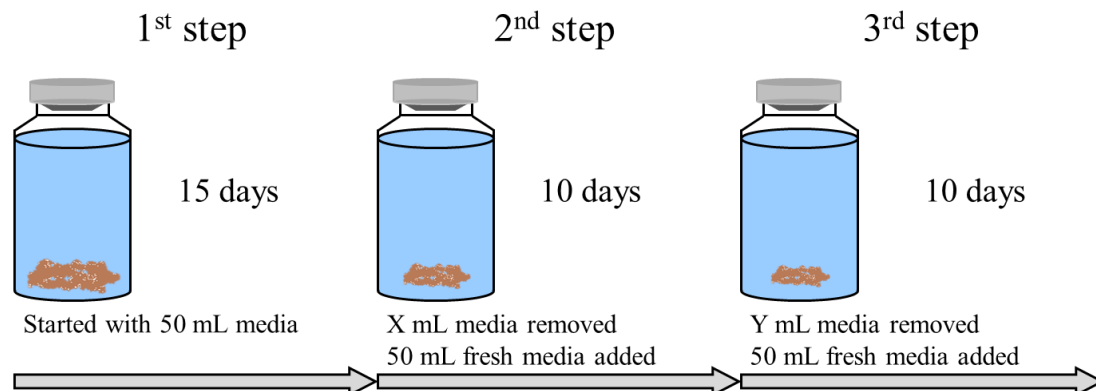


Fig. 5.50 Scheme of the experimental procedure of modified “three-step”.

In order to improve Ni recovery from the limonite ore, some of the leaching conditions were modified in this experiment. In the previous experiment (5.4.2.4), initial pH of each step was set at 1.2. Since 40% of Ca contents in the citrate washed limonite ore dissolved (via acid dissolution) at the beginning of the experiment, increase in pH values by Ca dissolution was not observed in 2nd and 3rd steps. Therefore, a bit higher initial pH of each step was set; 2nd and 3rd steps were started at initial pHs of pH 1.35 and 1.7, respectively. This may make microbial Fe(III) reduction of strains 7 and YTF1 active, since optimal pH for their growth is around 3.0. In order to prevent negative effect of increasing volume in head space of serum bottles (due to liquid sampling) on micro-aerobic conditions, leachate was replaced with 50 mL fresh media at each step.

Although, pH values increased to around 2.0 where strains 7 and YTF1 seem to be active, in both cell suspensions and inoculated cultures, changes in Fe and Ni dissolutions and solution redox potentials unexpectedly behaved similarly to those in the previous experiment: Final Ni recovery at 35 in cell suspensions and inoculated cultures was 44-49%, implying that Ni extraction efficiencies via reductive bioleaching at lower (45°C) and higher (70°C) temperatures were similar level. Maintenance of low redox potentials less than 300 mV is likely important for further increase in Ni recovery from the Indonesian limonite ore.

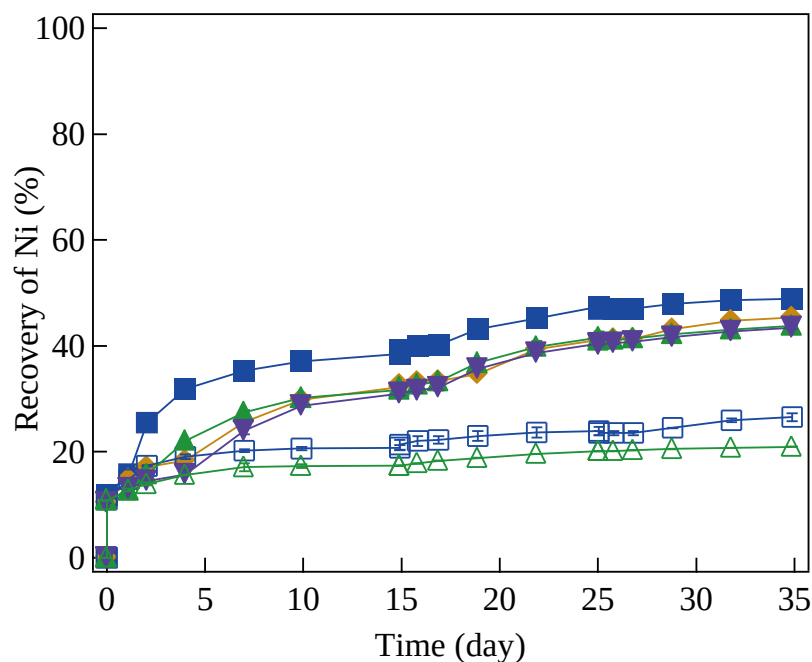


Fig. 5.51 Changes in the dissolution of total soluble Ni in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△).

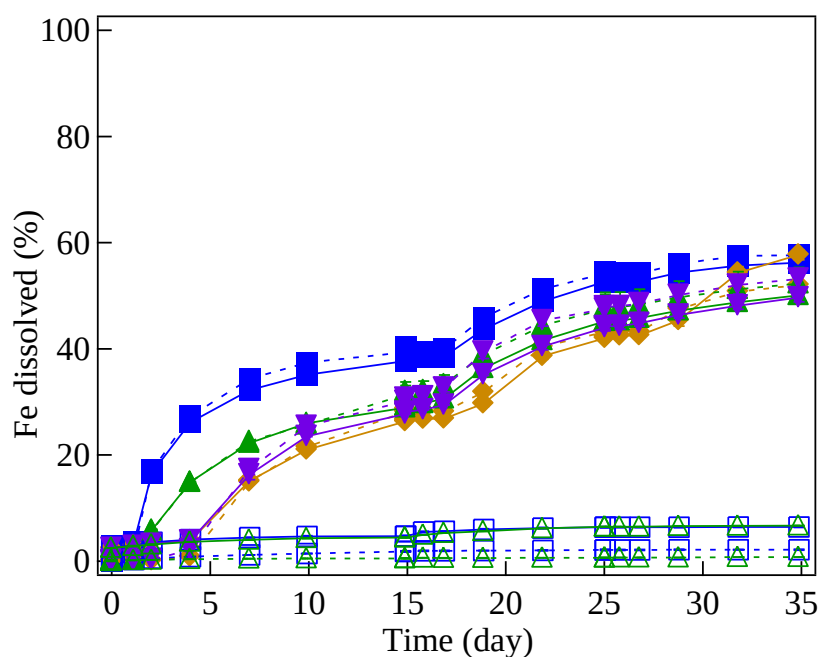


Fig. 5.52 Changes in the dissolutions of total soluble Fe (solid lines) and Fe(II) (broken lines) in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△).

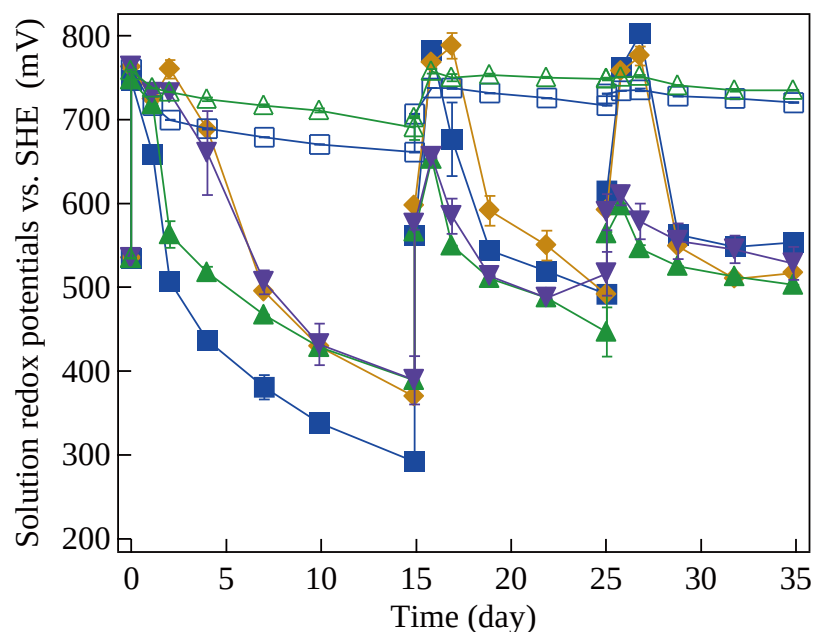


Fig. 5.53 Changes in the solution redox potentials in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△).

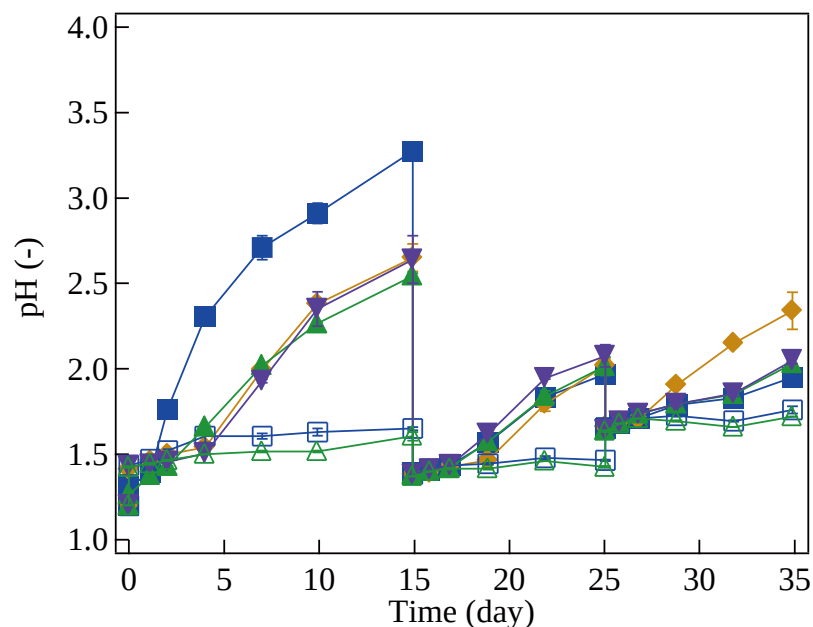


Fig. 5.54 Changes in the pH values in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△).

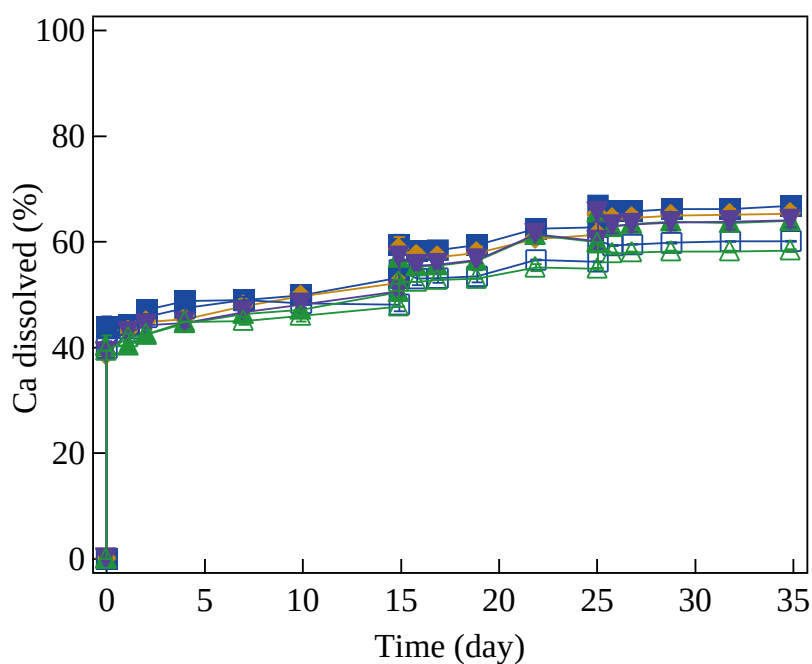


Fig. 5.55 Changes in the dissolution of total soluble Ca in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△).

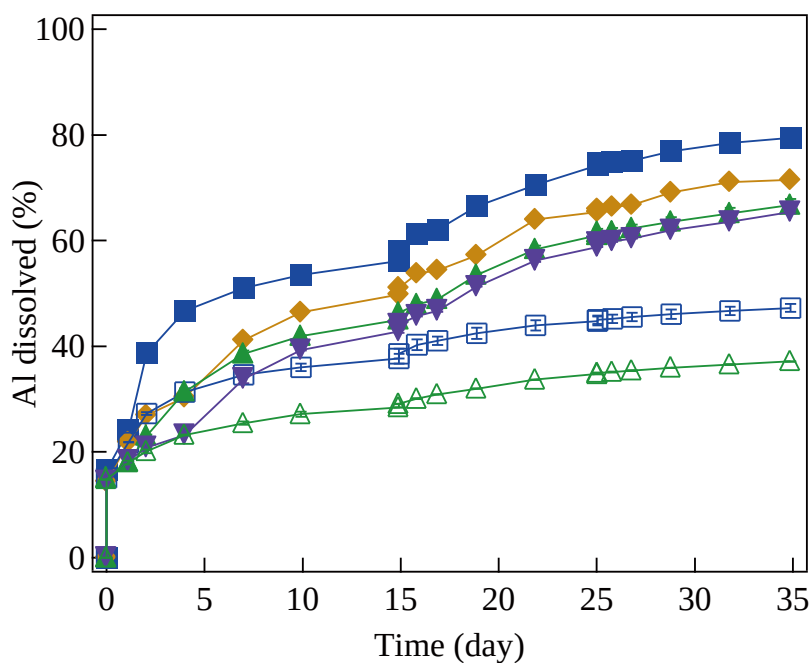


Fig. 5.56 Changes in the dissolution of total soluble Al in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△).

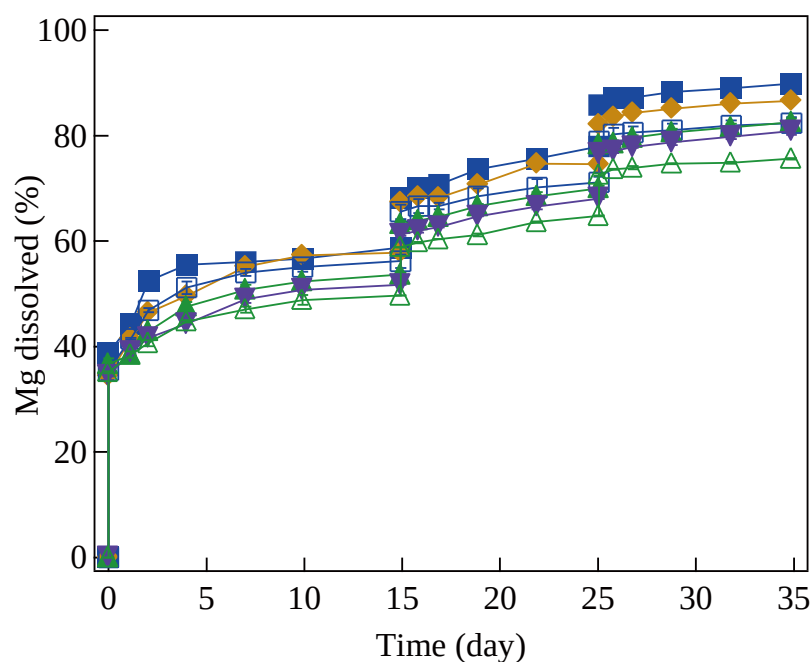


Fig. 5.57 Changes in the dissolution of total soluble Mg in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△).

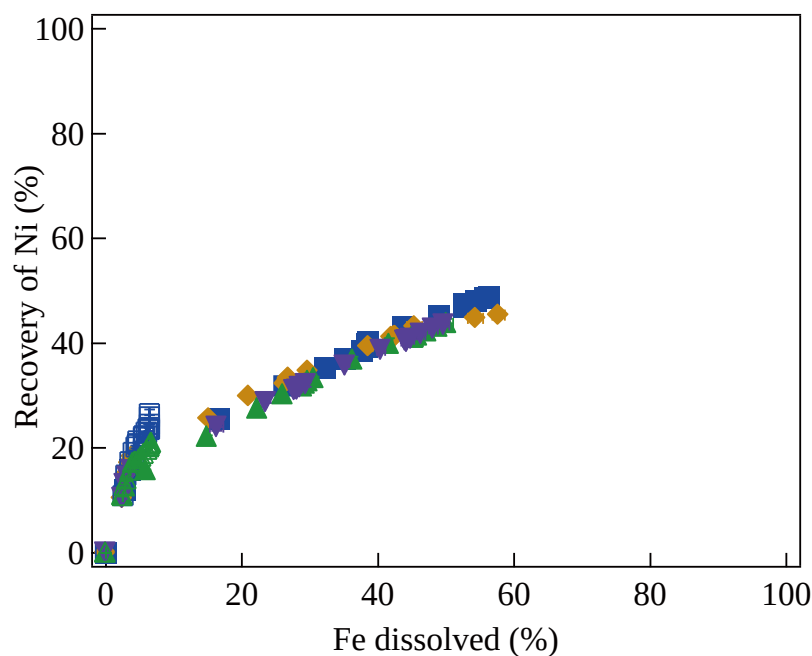


Fig. 5.58 The relationship between Ni dissolution and Fe dissolution in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△).

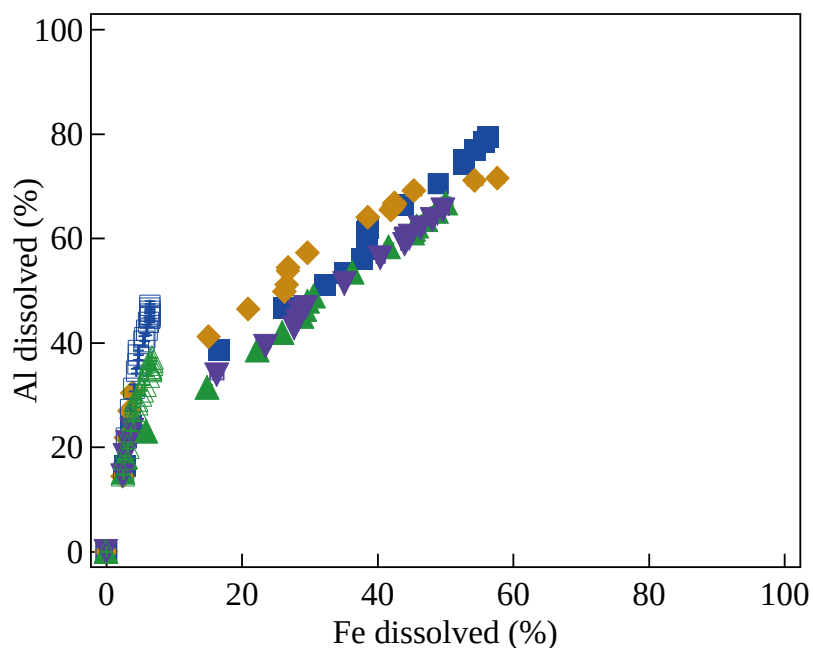


Fig. 5.59 The relationship between Al dissolution and Fe dissolution in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△).

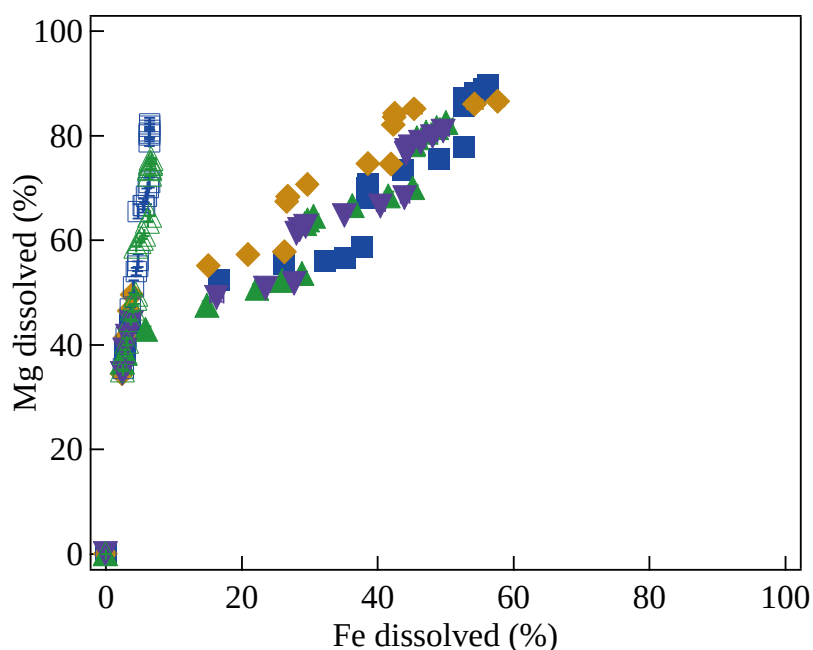


Fig. 5.60 The relationship between Mg dissolution and Fe dissolution in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△).

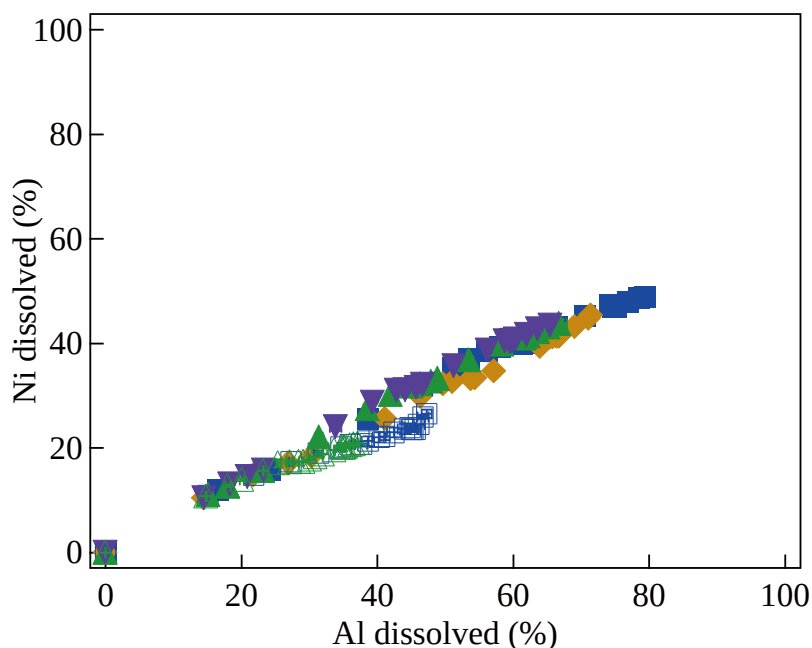


Fig. 5.61 The relationship between Ni dissolution and Al dissolution in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△).

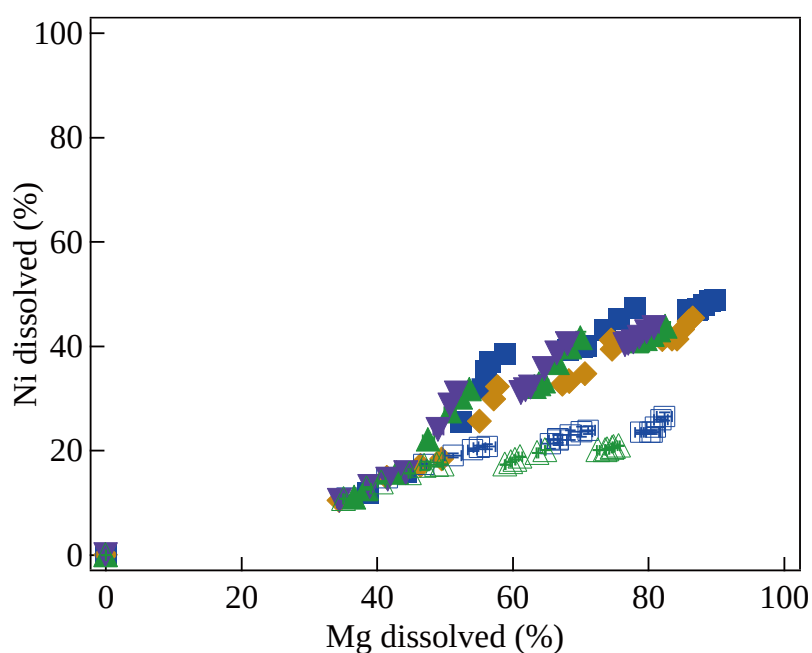


Fig. 5.62 The relationship between Ni dissolution and Mg dissolution in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△).

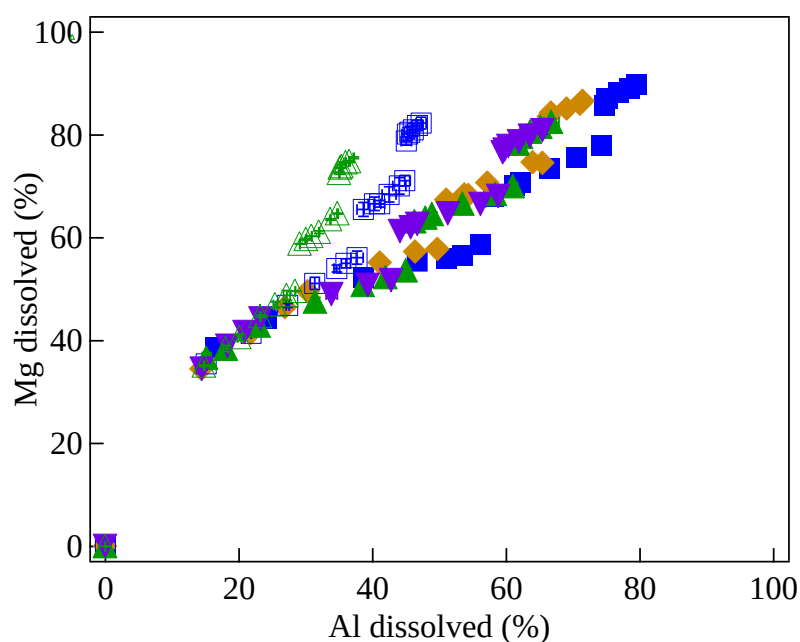


Fig. 5.63 The relationship between Mg dissolution and Al dissolution in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼) and in sterile controls at 70°C (□) or 45°C (△).

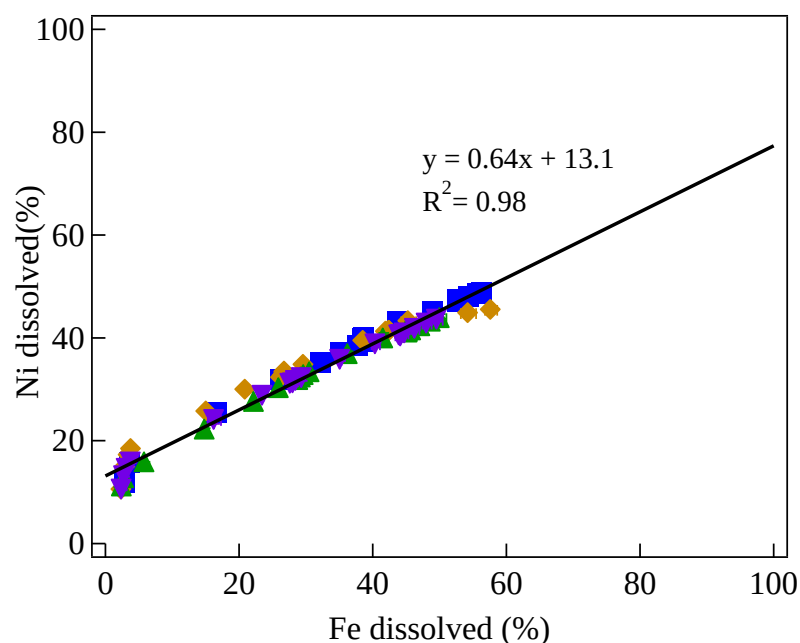


Fig. 5.64 Linear relationship between Ni dissolution and Fe dissolution in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼).

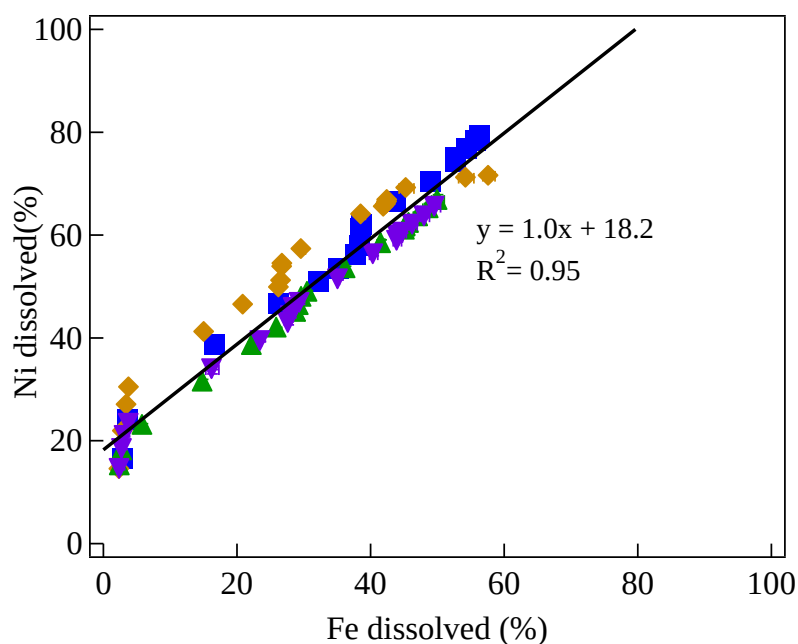


Fig. 5.65 Liner relationship between Al dissolution and Fe dissolution in cell suspensions (■ and ▲) and inoculated cultures (◆ and ▼) of *S. tokodaii* 7 (A; ■, B; ◆) or *Sb. acidophilus* YTF1 (A; ▲, B; ▼).

Table 5.9 Mineralogical composition of citrate washed limonite, residues in cell suspensions or inoculated cultures of *S. tokodaii* 7 and *Sb. acidophilus* YTF1, and in sterile controls (at 70 or 45°C), according to XRF analysis.

Elements mass %	Before bioleaching	After bioleaching					
	-	70°C			45°C		
	Citrate washed limonite	Cell suspensions of <i>S. tokodaii</i> 7	Inoculated cultures of <i>S. tokodaii</i> 7	Sterile controls	Cell suspensions of <i>Sb. acidophilus</i> YTF1	Inoculated cultures of <i>Sb. acidophilus</i> YTF1	Sterile controls
Fe	41.3	6.6	17.4	43.3	19.0	17.3	42.3
Si	12.3	37.4	29.5	12.6	28.0	29.1	13.2
Al	1.9	1.5	1.7	1.64	2.0	2.1	1.9
Mg	3.0	1.8	1.7	1.1	2.2	2.1	1.2
Cr	1.5	1.67	2.37	1.8	1.63	1.87	1.5
Ni	2.10	0.21	0.44	1.15	0.65	0.59	1.3
S	< 10 µg/g	0.42	0.29	0.35	0.34	0.38	0.30
Mn	0.31	0.20	0.22	0.24	0.10	0.08	0.24
Ca	0.48	0.26	0.24	0.17	0.24	0.25	0.15
P	0.037	0.055	0.192	0.259	0.123	0.120	0.201
V	0.022	0.010	0.011	0.024	0.008	0.009	0.023
Zn	0.037	0.020	0.024	0.026	0.025	0.022	0.025
K	< 1 µg/g	0.12	0.084	0.036	0.072	0.10	0.018
Sr	< 0.1 µg/g	< 0.1 µg/g	0.00	< 0.1 µg/g	0.00	0.00	< 0.1 µg/g
Ti	< 1 µg/g	0.21	0.21	< 1 µg/g	0.19	0.19	< 1 µg/g
Br	< 0.1 µg/g	< 0.1 µg/g	< 0.1 µg/g	< 0.1 µg/g	0.0085	0.0070	< 0.1 µg/g
Cu	< 1 µg/g	0.016	0.018	< 1 µg/g	0.030	0.010	< 1 µg/g

5.5 Conclusions

Screening of useful Fe(III)-reducing acidophiles was carried out via reduction capability of soluble Fe(III) and dissolution capability of chemically synthesized goethite in section 1, resulting in selection of representative Fe(III)-reducing acidophiles including *Sb. acidophilus* YTF1 (moderately thermophilic acidophile), *S. tokodaii* 7 and *S. shibae* B12 (both thermophilic acidophiles). Although acid washed-Indonesian limonite ore was applied to reductive bioleaching using Fe(III)-reducing acidophiles, the difference in Ni recovery between cell suspensions and sterile controls was only marginal, which was caused by the increase in pHs by dissolution of Ca contents in limonite ore. In order to remove Ca contents in limonite ore, the raw limonite ore was washed with 1 M citrate, and then the citrate washed-limonite was used to the following reductive bioleaching. Dissolution of goethite phase in limonite ore used in this study was indirectly promoted by microbial Fe(III) reduction by *Sb. acidophilus* YTF1 and *S. tokodaii* 7. Reductive dissolution at higher temperature (70°C) was found to result in higher Fe dissolution and also associated Ni extraction. Furthermore, even though initial cell density was increased from 1×10^7 cells/mL to 1×10^9 cells/mL, final Ni extraction at 30 days was not significantly affected (improved from 57 to 64% using *S. tokodaii* 7).

Ni extraction via Fe dissolution from the Indonesian limonite ore was found to be sensitive to solution redox potential levels, total soluble Fe concentrations, and solution pHs. Since one of the limit factors to Ni extraction was a decrease in solubility capacity of total soluble Fe at higher pHs (The reaction speed of goethite dissolution declined due to a decrease in Fe solubility capacity in leachate), Ni extraction via goethite dissolution was found to increase by refreshing bioleaching media (repeating reductive bioleaching). The maximum Ni extraction within 30 days (55%) was marked in the three-step reductive bioleaching.

In this chapter, improvement in Ni recovery from Indonesian limonite ore via reductive bioleaching using microbial Fe(III)-reducing acidophiles was investigated, and its potential applicability to practical operation for Ni recovery was shown. Further studies on optimization of the bioleaching conditions (e.g., anaerobic/micro-aerobic conditions, pulp densities, initial pHs, bioreactor/column experiments, etc.) will contribute to development of Ni recovery techniques from limonitic laterite ores.

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The results in this chapter were partially cooperated by Mr. Katsutoshi Tsutsumi (master 2 student in fiscal year 2016).

Chapter 6

Applicability of reductive bioleaching of refractory chalcopyrite using Fe(III)-reducing acidophiles

Abstract

Reductive bioleaching of ferric oxide ores such as Ni associated-limonite using microbial Fe(III)-reducing activity at acidic pHs under micro-aerobic/anaerobic conditions has recently been studied. The metal dissolution technique may be able to be applicable to not only ferric oxides, but also sulfide minerals including chalcopyrite (CuFeS_2). In general, bioleaching of sulfide minerals has been studied via the oxidative bioleaching, using Fe(II)- and/or S^0 -oxidizing acidophiles under aerobic conditions. However, the reductive bioleaching approach has yet to be successful for “refractory” sulfide minerals. This study therefore investigated the applicability of the reductive bioleaching approach for sulfide minerals, especially chalcopyrite. Since microbial Fe(III)-reducing capabilities under micro-aerobic/anaerobic conditions have been confirmed with some *Sulfolobus* spp. with optimum pH and temperature of 2.0 and 70°C, respectively, strains in the genus *Sulfolobus* was used for reductive bioleaching of chalcopyrite. However, no noticeable effect on Cu extraction was shown in reductive bioleaching of chalcopyrite, indicating that shift of chemical equilibrium of Fe(III) between solid and solution phase is invalid for Cu extraction from refractory chalcopyrite since its refractoriness are too high to solubilize chalcopyrite.

6.1 Introduction

Bioleaching of sulfide minerals (e.g., chalcopyrite (CuFeS_2), enargite (Cu_3AsS_4), pyrite (FeS_2)) has generally been studied with Fe(II)- and/or S^0 -oxidizing acidophiles at acidic pHs under aerobic conditions (Schippers et al 1996, Yahya et al 2002, Rodríguez et al 2003, Zhang et al 2008, Vilcáez et al 2008, Fu et al 2008, Gautier et al 2008, Karimi et al 2010, Sasaki et al 2010, Zhu et al 2011, Sasaki et al 2011, Takatsugi et al 2011,). However, so far, reductive bioleaching of sulfide minerals has yet been reported. Since chalcopyrite (CuFeS_2) contains Fe in the form of ferric (in fact, not exactly as ferric) in its crystal structure, this chapter aimed to investigate applicability of direct reduction of Fe(III) in chalcopyrite solid phase to soluble Fe(II) using Fe(III)-reducing acidophiles, as follows,

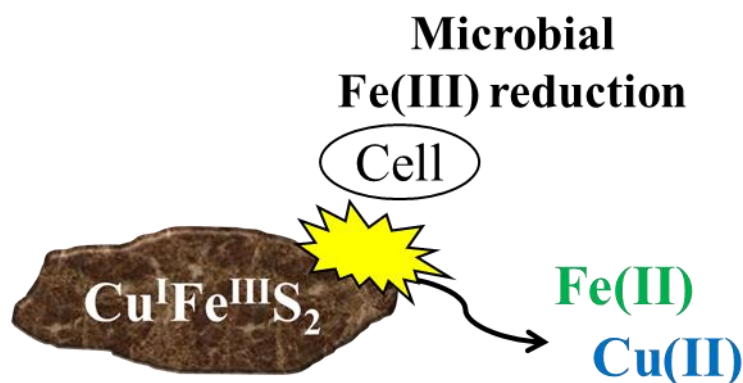
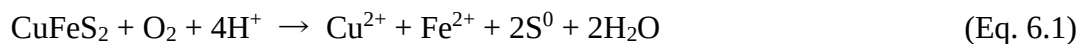


Fig. 6.1 Scheme of the expected mechanism of reductive bioleaching of chalcopyrite vis microbial Fe(III) reduction.

In the case of oxidative (conventional) bioleaching of sulfide minerals, continuous aeration to the bioleaching system is necessary in order to maintain sufficient dissolved oxygen levels since oxygen is consumed by dissolution of sulfide minerals, microbial oxidation of Fe(II) and S^0 as shown in Eq. 6.1-6.3.



Moreover, addition of acid, generally sulfuric acid, to the chemical leaching system is also important to maintain solution pHs low to avoid forming ferric precipitates. The addition of net acidity is generally not required in the case of bioleaching system since use of S^0 -oxidizing acidophiles produce protons through oxidation of S^0 to sulfate as shown in Eq. 6.3.

On the other hand, reductive bioleaching system of sulfide minerals does not require the additional supply of air (oxygen), since Fe(III)-reducing acidophiles generally prefer micro-aerobic or anaerobic to aerobic conditions for their growths and microbial Fe(III)-reducing activities. This can surely be advantages for less cost-industrial operations of Cu extraction from Cu-containing sulfide minerals. Therefore, investigation of applicability of reductive bioleaching of chalcopyrite can contribute to future development in Cu extraction process and provide another choice for metal extraction process on site.

In this chapter, *Sulfolobus* spp. was selected as microbial Fe(III) reducers. As was described in chapter 5, some *Sulfolobus* strains, especially *S. shibatae* B12 and *S. tokodaii* 7, were found to effectively reduce soluble Fe(III) to Fe(II) under micro-aerobic and anaerobic conditions. The use of thermophilic (optimum temperature of 75-80°C) acidophiles may result in relatively faster reactions since the efficiency of the oxidative bioleaching of sulfide minerals by thermophiles was reported to be more significant than that by mesophiles (Rodriguez et al 2003). When the oxidative bioleaching process is operated in heaps, the microbial system is expected to grow at high temperature at least 70°C since temperature inside heap is, in general, around 70-80°C (Petersen et al 2002). Therefore, use of thermophilic acidophiles is reasonable when considering the operational application such as heaps.

6.2 Materials and methods

6.2.1 Reductive bioleaching of chalcopyrite using inoculated cultures or cell suspensions of *Sulfolobus* spp. under micro-aerobic conditions

Cells of *S. shibatae* B12, *S. tokodaii* 7 and *Sulfolobus* sp. GA1 were pre-grown, harvested at the late-exponential phase by centrifugation (12,000 g, 10 min at 40°C), washed twice with flesh HBS medium (pH 2.0 with H₂SO₄). All the reductive bioleaching tests were carried out in 50 mL serum bottles containing 50 mL N₂-purged (< 2.9 mg/L) HBS medium (pH 2.0 with H₂SO₄) N₂-purged 10 mM glucose and 0.01% yeast extract as electron donor and carbon sources, respectively. The initial pulp density of acid washed-chalcopyrite concentrate was set to 2% (w/v). The initial cell density was set to 1×10⁷ or 1×10⁹ cells/mL. All the media were maintained at 70°C on an orbital rotary shaker at 120 rpm. Samples were regularly taken using syringe needles to monitor cell density, pH values, solution redox potential values, and concentrations of Fe(II), Cu(I), total soluble Fe and Cu. All the experiments were carried out in duplicates.

6.2.2 Reductive bioleaching of chalcopyrite using mixed culture of *Sulfolobus* spp.

Cells of *S. metallicus* Kra23, *S. acidocaldarius* 98-3, *S. solfataricus* P1, *S. tokodaii* 7, and *S. shibatae* B12 were pre-grown, harvested at the late-exponential phase by centrifugation (12,000 g, 10 min at 40°C), washed twice with flesh HBS medium (pH 1.1 with H₂SO₄). All the reductive bioleaching tests were carried out in 10 mL serum bottles containing 100 mL HBS medium (pH 1.1 with H₂SO₄; un-purged with N₂) with 0.01% (w/v) yeast extract, 0.01% (w/v) elemental sulfur, 5 mM Fe(II) and 5 mM Cu(II). The initial pulp density of acid washed-chalcopyrite concentrate was set to 1% (w/v). The initial cell density was set to 1×10⁷ cells/mL for each strain (5×10⁷ cells/mL in total). All the media were maintained at 70°C on an orbital rotary shaker at 130 rpm. Samples were regularly taken using syringe needles to monitor cell density, pH values, solution redox potential values, and concentrations of Fe(II), total soluble Fe and Cu. All the experiments were carried out in duplicates.

6.3 Results and discussion

6.3.1 Reductive bioleaching of chalcopyrite using inoculated cultures or cell suspensions of *Sulfolobus* spp. under micro-aerobic conditions

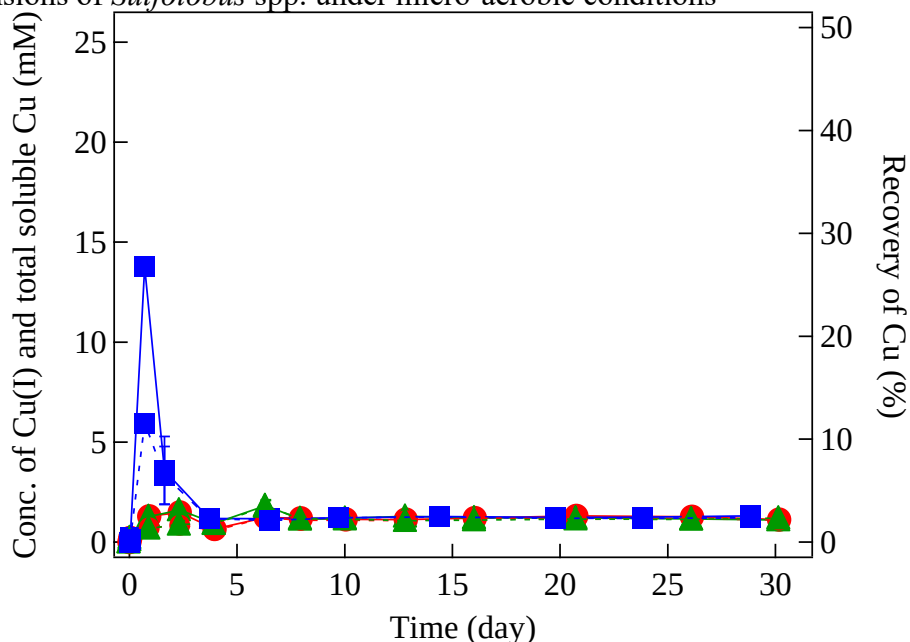


Fig. 6.2 Changes in the concentrations of total soluble Cu (solid lines) and Cu(I) (broken lines) in inoculated cultures of *S. shibatae* B12 (●, ○), *S. tokodaii* 7 (■, □) and *Sulfolobus* sp. GA1 (▲, △) under micro-aerobic conditions.

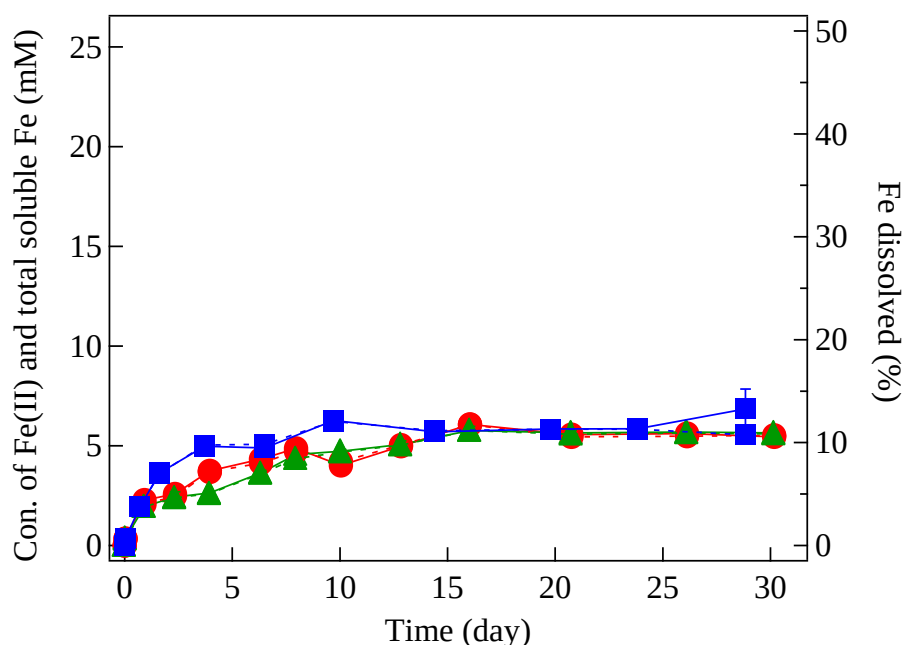


Fig. 6.3 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) in inoculated cultures of *S. shibatae* B12 (●, ○), *S. tokodaii* 7 (■, □) and *Sulfolobus* sp. GA1 (▲, △) under micro-aerobic conditions.

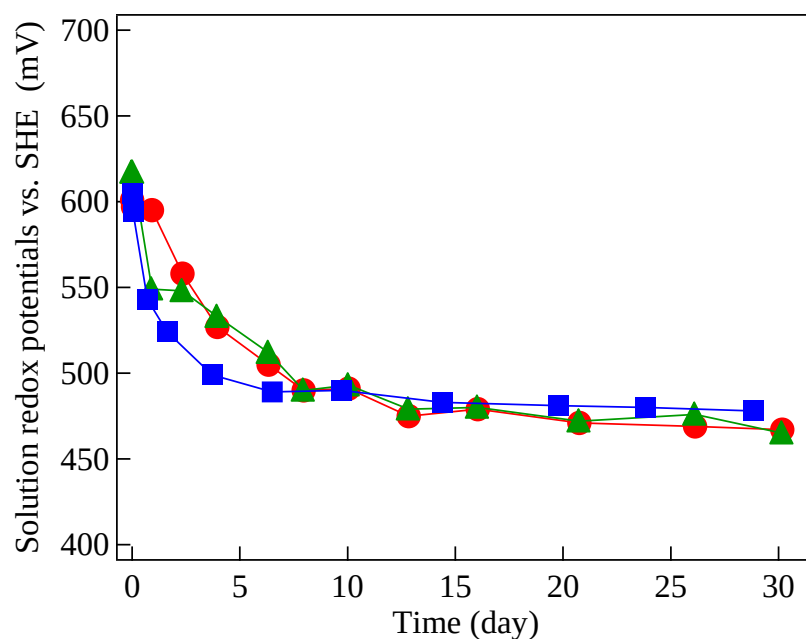


Fig. 6.4 Changes in the solution redox potentials in inoculated cultures of *S. shibatae* B12 (●, ○), *S. tokodaii* 7 (■, □) and *Sulfolobus* sp. GA1 (▲, △) under micro-aerobic conditions.

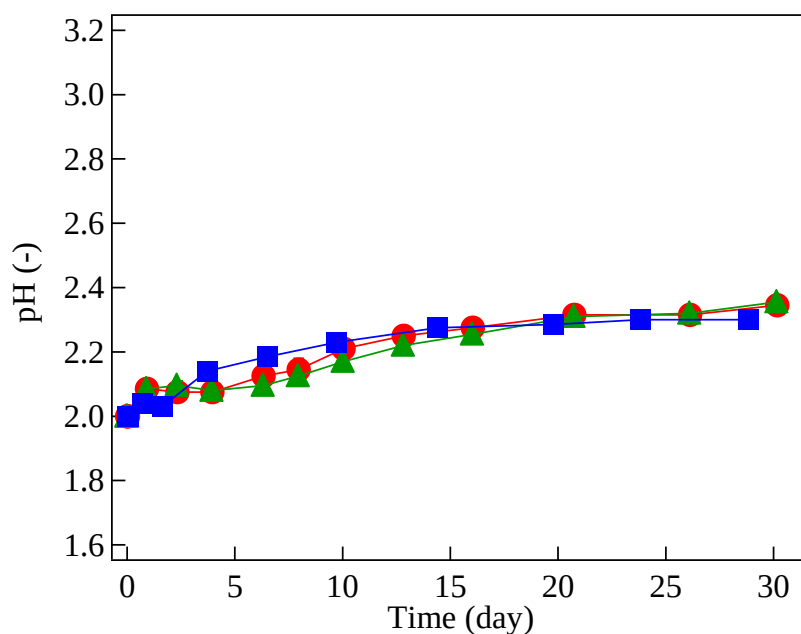


Fig. 6.5 Changes in the pHs in inoculated cultures of *S. shibatae* B12 (●, ○), *S. tokodaii* 7 (■, □) and *Sulfolobus* sp. GA1 (▲, △) under micro-aerobic conditions.

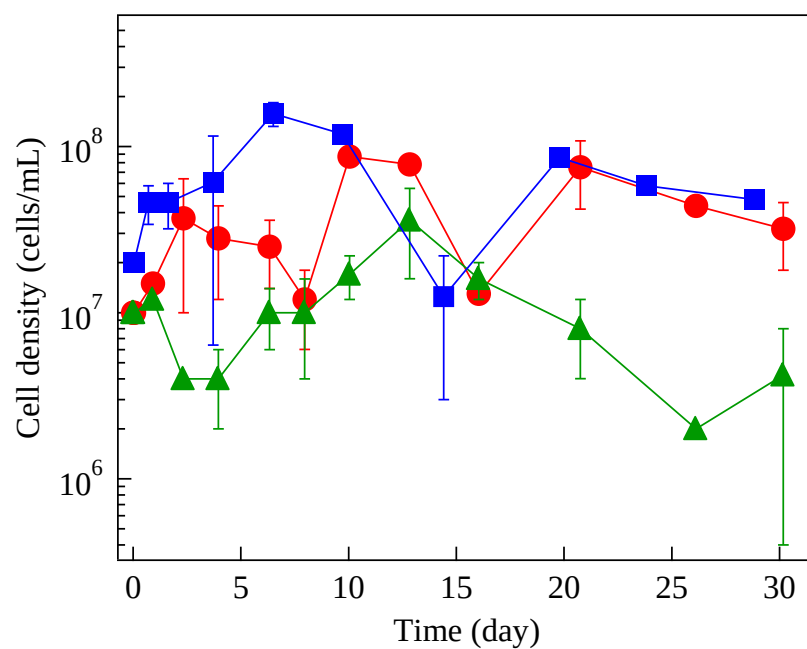


Fig. 6.6 Changes in the cell density in inoculated cultures of *S. shibatae* B12 (●, ○), *S. tokodaii* 7 (■, □) and *Sulfolobus* sp. GA1 (▲, △) under micro-aerobic conditions.

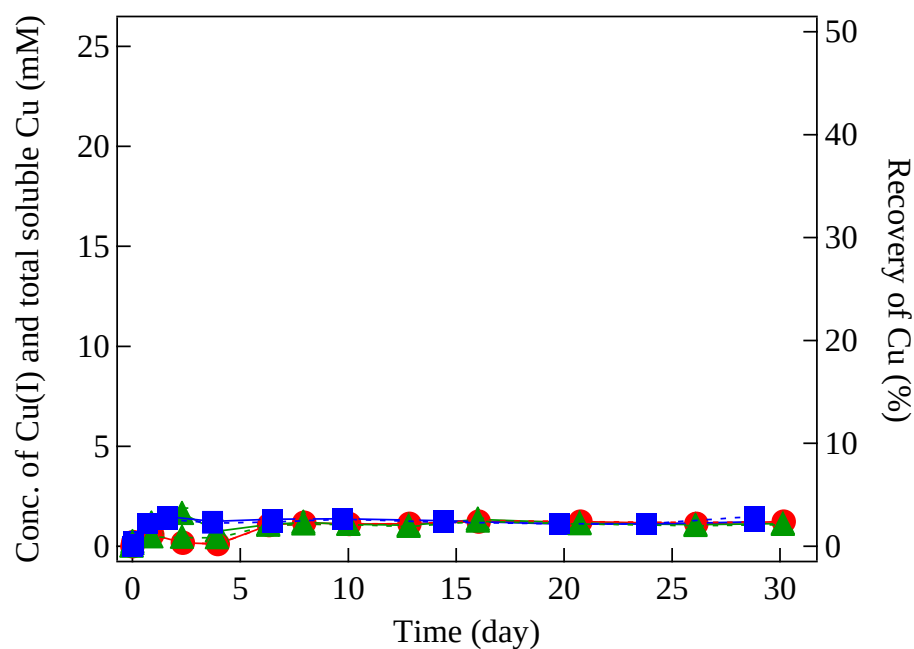


Fig. 6.7 Changes in the concentrations of total soluble Cu (solid lines) and Cu(I) (broken lines) in cell suspensions of *S. shibatae* B12 (●, ○), *S. tokodaii* 7 (■, □) and *Sulfolobus* sp. GA1 (▲, △) under micro-aerobic conditions.

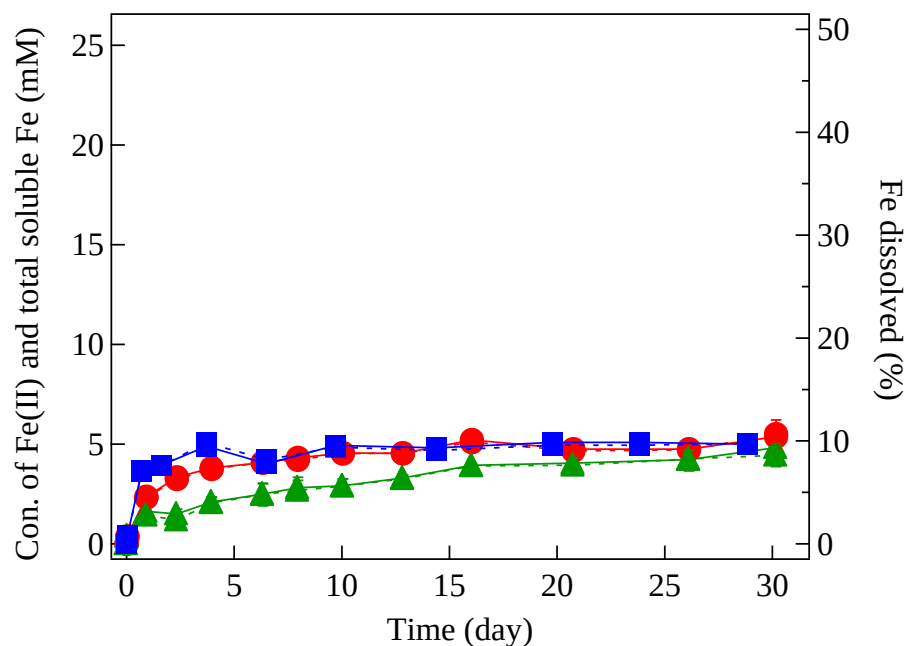


Fig. 6.8 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) in cell suspensions of *S. shibatae* B12 (●, ○), *S. tokodaii* 7 (■, □) and *Sulfolobus* sp. GA1 (▲, △) under micro-aerobic conditions.

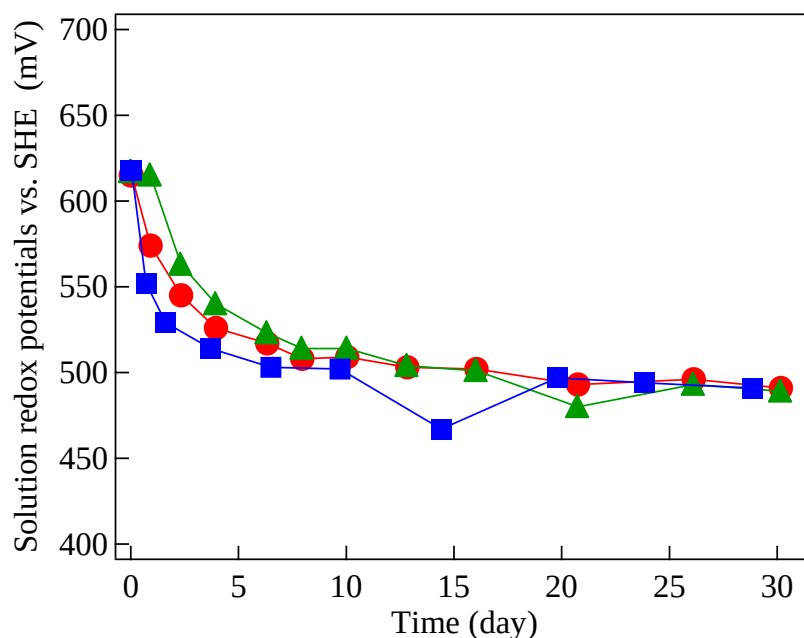


Fig. 6.9 Changes in the solution redox potentials in cell suspensions of *S. shibatae* B12 (●, ○), *S. tokodaii* 7 (■, □) and *Sulfolobus* sp. GA1 (▲, △) under micro-aerobic conditions.

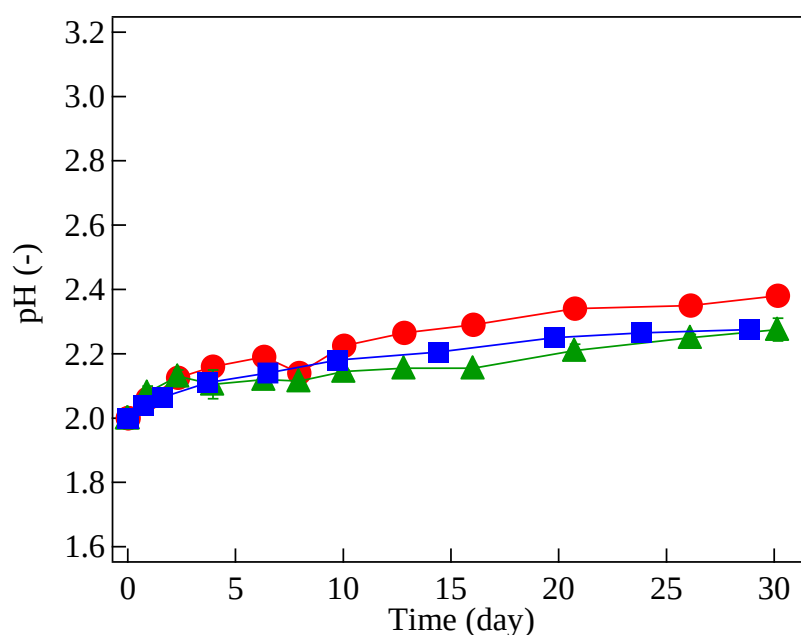
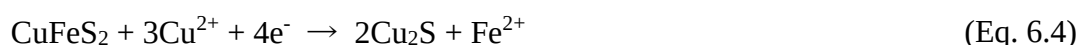


Fig. 6.10 Changes in the pHs in cell suspensions of *S. shibatae* B12 (●, ○), *S. tokodaii* 7 (■, □) and *Sulfolobus* sp. GA1 (▲, △) under micro-aerobic conditions.

Utilization of *Sulfolobus* strains did not succeed in Cu extraction from chalcopyrite. Cu recovery and Fe dissolution in inoculated cultures at day 30 were only marginal, approximately 2% and 10%, respectively: Cu extraction was ineffective in all inoculated cultures throughout the experiment. The concentration of total soluble Fe gradually increased up to over 5 mM with Fe(II) as dominant ion species, resulting in decrease in solution redox potentials. Although the concentration of total soluble Cu was constant at around 1 mM throughout the experiment (after its initial sudden increase at day 1), approximately 5.5 mM total soluble Fe dissolved until at least day 15. In general, chalcopyrite consists of Fe and Cu in same molar ratio (1:1), theoretically indicating that the concentrations of Fe and Cu dissolved in the chalcopyrite dissolution should be same. Therefore, it is possible that chalcopyrite is transferred to chalcocite as shown in Eq. 6.4.



Even if this is the case, oxidation of chalcocite does not occur since solution redox

potentials decreased from 600 to 480 mV within 30 days, probably owing to microbial Fe(III)-reducing capability.

As for pH values, it gradually increased as chalcopyrite dissolved. This phenomenon is common for dissolution of sulfide minerals as was mentioned in the chapter of literature review. Overall, reductive bioleaching of chalcopyrite in inoculated culture of Fe(III)-reducing acidophiles seemed not to be effective. In the next experiment, cell suspensions of the three *Sulfolobus* strains was used in order to maximize microbial activity of Fe(III) reduction.

No improvement in Cu recovery and Fe dissolution was observed compared with those in the previous experiment. XRD patterns of residual minerals also showed no formation of secondary products such as jarosite and elemental sulfur in the inoculated cultures and cell suspensions. These two experiments regarding reductive bioleaching of chalcopyrite using Fe(III)-reducing ability indicated its ineffectiveness compared with the conventional oxidative bioleaching.

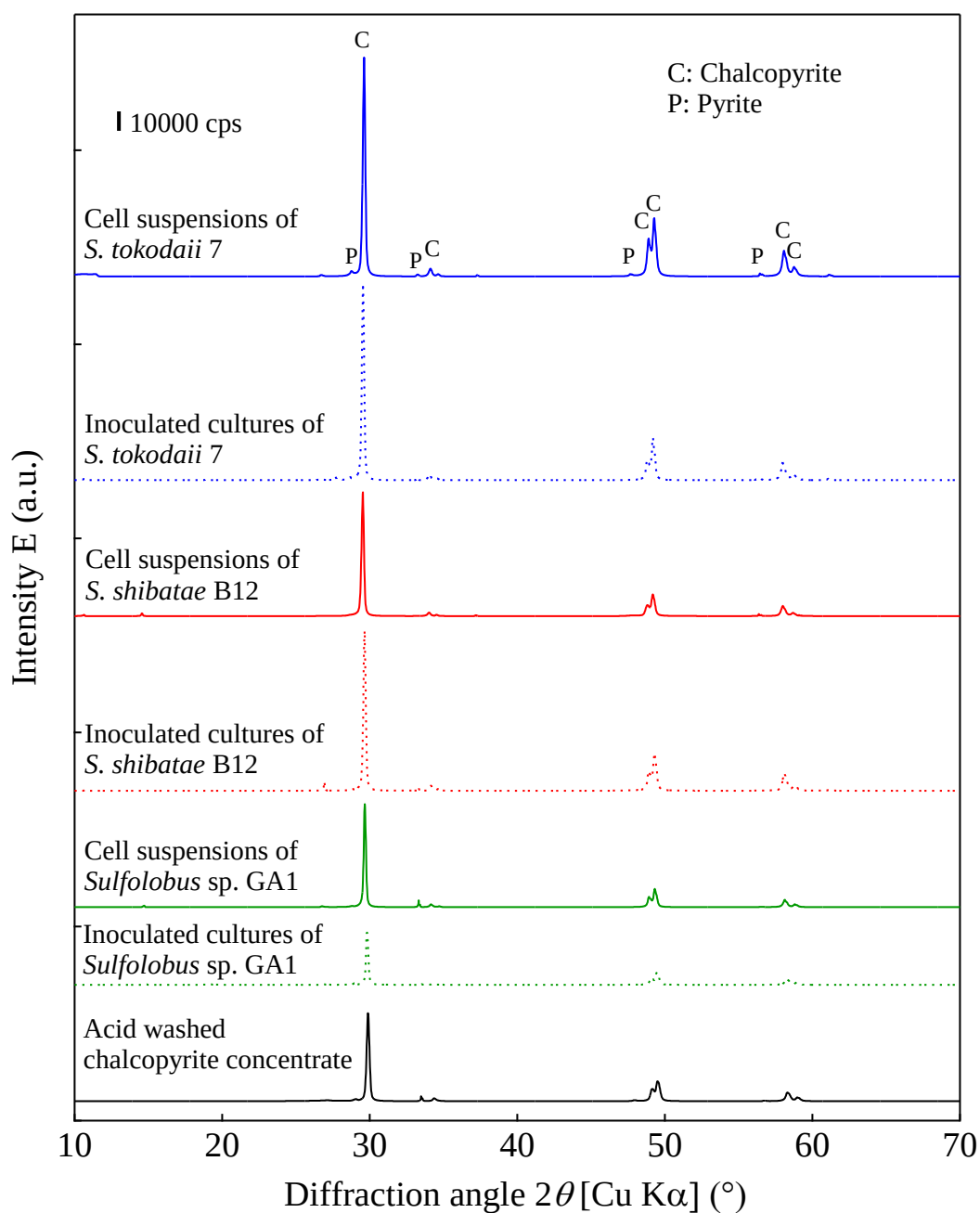


Fig. 6.11 X-ray diffraction patterns of original chalcopyrite concentrate, residual mineral in inoculated cultures and cell suspensions of *Sulfobolus* sp. GA1, *S. shibatae* B12, and *S. tokodaii* 7, respectively, from the bottom to the top. “C” and “P” indicate the peak patterns corresponding to chalcopyrite and pyrite, respectively.

6.3.2 Reductive bioleaching of chalcopyrite using mixed culture of *Sulfolobus* spp.

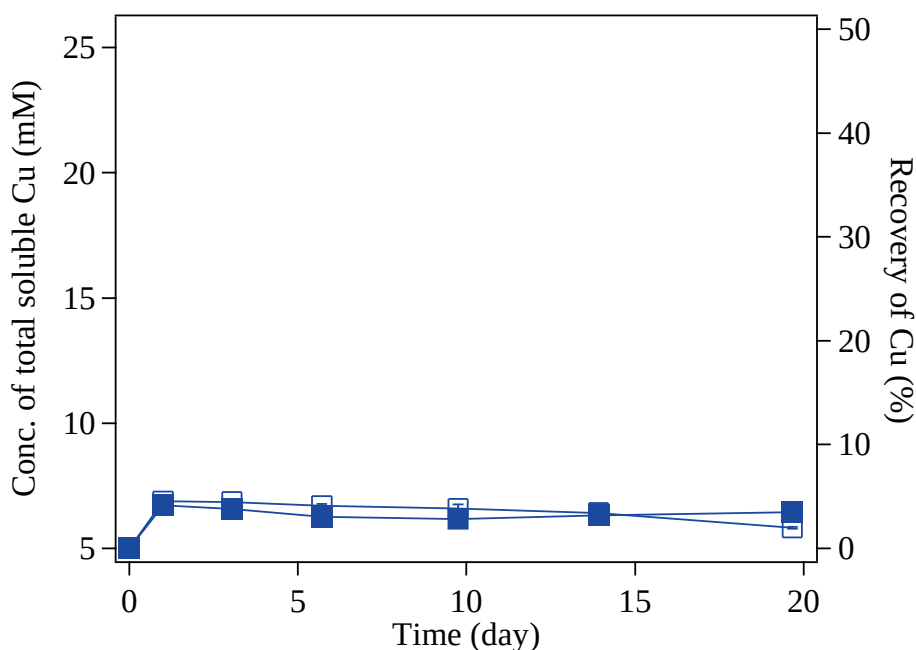


Fig. 6.12 Changes in the concentrations of total soluble Cu in inoculated cultures (■) and sterile controls (□) under micro-aerobic conditions.

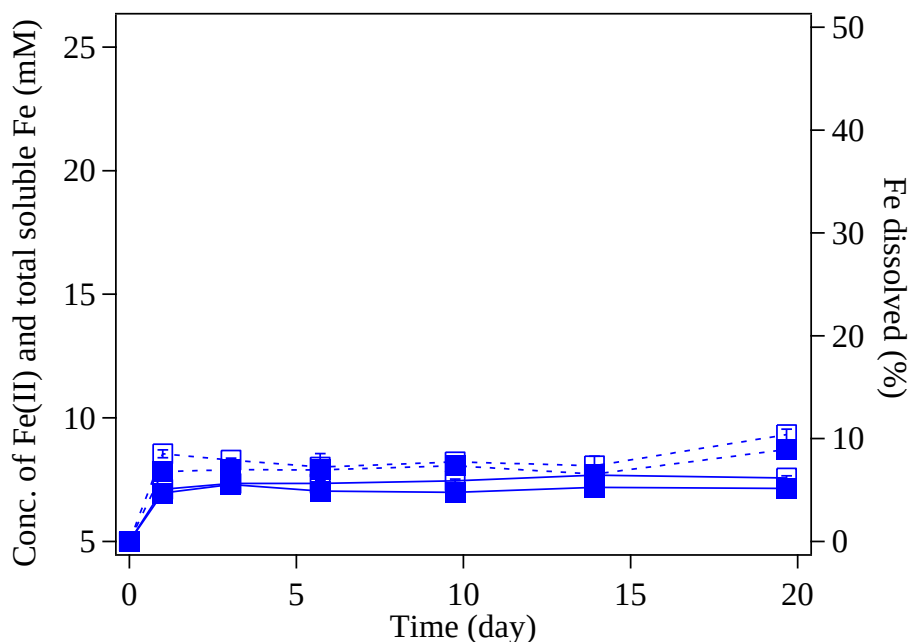


Fig. 6.13 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) in inoculated cultures (■) and sterile controls (□), respectively, under micro-aerobic conditions.

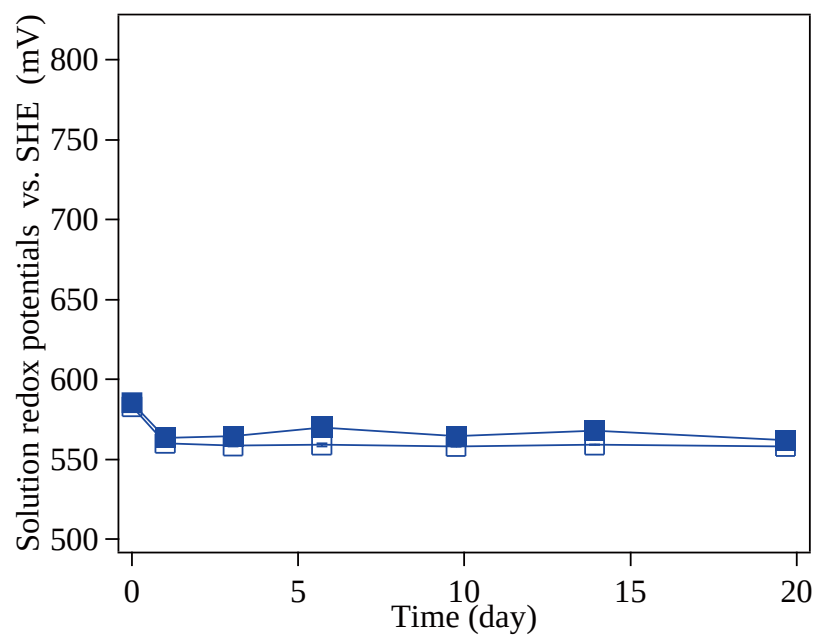


Fig. 6.14 Changes in the solution redox potentials in inoculated cultures (■) and sterile controls (□) under micro-aerobic conditions.

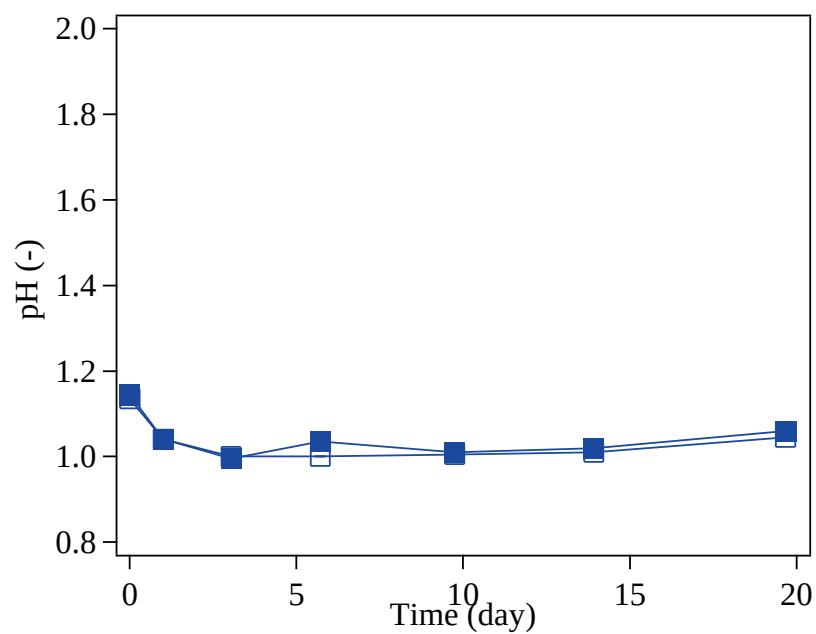


Fig. 6.15 Changes in the pHs in inoculated cultures (■) and sterile controls (□) under micro-aerobic conditions.

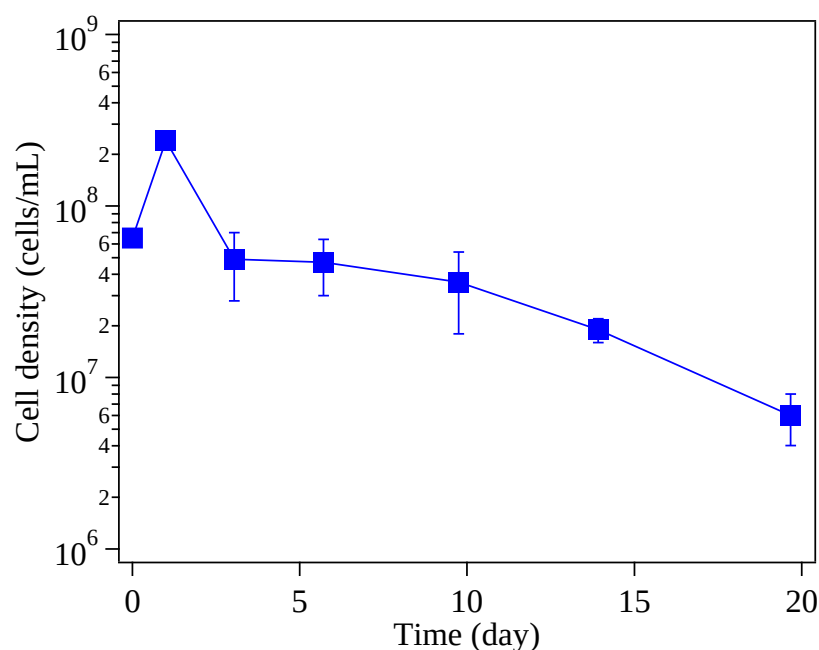


Fig. 6.16 Changes in the cell density in inoculated cultures (■) under micro-aerobic conditions.

Regardless of the presence of microorganisms, dissolution of Cu and Fe was only confirmed at day 1 by acid dissolution. However, after that, no more Cu recovery and Fe dissolution were observed throughout the experiment, clearly indicating that reductive bioleaching of chalcopyrite via microbial Fe(III) reduction is unlikely available for Cu extraction from chalcopyrite. The reason for the results may be higher refractoriness of chalcopyrite. Therefore, reductive bioleaching technique was found not to be applicable to refractory primary sulfides, chalcopyrite.

6.4 Conclusions

In this chapter, applicability of reductive bioleaching technique to Cu recovery from refractory primary sulfides, chalcopyrite, was investigated using *Sulfolobus* spp. as Fe(III)-reducing acidophiles. However, no noticeable effect on Cu extraction was shown in reductive bioleaching of chalcopyrite in this chapter. Therefore, these experiments in this chapter regarding reductive bioleaching of chalcopyrite revealed that direct reduction of Fe(III) in chalcopyrite structure to soluble Fe(II) using microbial Fe(III)-reducing capability unlikely occurs, due to its high refractoriness.

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Chapter 7

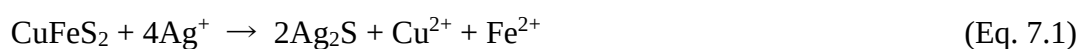
Enhancement of Cu recovery via oxidative bioleaching of refractory chalcopyrite in the presence of silver catalyst

Abstract

Cu recovery in both bio and chemical leaching systems of chalcopyrite has been reported to be improved by the addition of small amount of Ag catalyst in the forms of Ag_2SO_4 , Ag_2S , or AgCl . In this chapter, the effect of addition of Ag catalyst on the chalcopyrite bioleaching system was investigated using mixed cultures of moderately thermophilic acidophiles (Fe(II)-oxidizers and S^0 -oxidizer) at different initial pHs. The effect of the addition of Ag(I) (in section 1) and Ag_2S (in section 2) to bioleaching systems on Cu recovery was evaluated. In mixed cultures, the addition of Ag catalyst resulted in inhibitory effect on microbial Fe(II)- and S^0 -oxidizing activities. After the addition of Ag catalyst, solution redox potentials in sterile controls fell down to a level where chalcopyrite dissolution can be facilitated, due to accumulation of Fe(II) as product of chalcopyrite dissolution through Ag catalyzed reactions, resulting in higher Cu recovery in sterile controls than that in mixed cultures. The results obtained in this chapter indicate that a balance between Fe(II) accumulation via Ag catalyzed leaching of chalcopyrite and microbial Fe(II)-oxidizing activity that can maintain the E_{normal} levels at the specific range ($0 < E_{\text{normal}} < 1$), is important in order to maximize Cu recovery in Ag catalyzed-bioleaching system.

7.1 Introduction

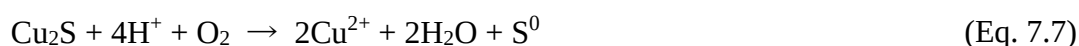
Enhancement in Cu recovery from primary copper sulfide minerals (e.g., chalcopyrite) has actively been investigated. Cu recovery in both bio and chemical leaching systems of chalcopyrite was reported to be improved by the addition of small amount of Ag catalyst in the forms of Ag₂SO₄, Ag₂S, or AgCl (Mier et al 1995, Sato et al 2000, Hiroyoshi et al 2002, López-Juárez et al 2006, Muñoz et al 2007, Deng et al 2009). The proposed mechanism of chalcopyrite dissolution through Ag catalytic reaction is shown below;



As shown in Eq. 7.2, Fe(III) is necessary for re-generation of Ag(I) from Ag₂S, indicating that microbial Fe(II)-oxidizing capability is likely able to facilitate Fe(III) re-production in bioleaching system. Furthermore, additional use of S⁰-oxidizing acidophiles contributes to suppression of S⁰ accumulation onto chalcopyrite surface. Therefore, the experiments in chapter 7 were carried out (1) to evaluate effect of silver catalyst on chalcopyrite bioleaching using moderately thermophilic acidophiles and (2) to investigate its detailed mechanism.

Other research groups reported that catalytic effect of Ag addition on chalcopyrite bio/chemical leaching depends on concentration of Ag(I) (Hiroyoshi et al 2002, Muñoz et al 2007). As shown in Fig. 7.1, Cu extraction rate at higher Ag(I) concentration is theoretically higher compared with that at lower Ag(I) concentration. Chalcopyrite leaching mechanism follows Eq. 7.1 and 7.2 at higher Ag(I) concentration. Due to an actual range of solution redox potentials in chalcopyrite leaching system (as is described in Fig. 7.1), Cu extraction rate increases with increasing Ag(I) concentration. At lower Ag(I) concentration, chalcocite formation and its oxidation are involved in chalcopyrite leaching mechanism at solution redox potentials between E_{ox} and $E_c(\text{Ag}^+)$ as follows;





Since Cu extraction rate at lower Ag(I) concentration is maximized at solution redox potentials between E_{ox} and $E_c(\text{Ag}^+)$, increase in solution redox potential does not lead to further increase in Cu extraction rate. Considering the fact that silver compounds often inhibit microbial activity, the silver catalyzed-chalcopyrite leaching model at lower Ag(I) concentration, but not higher, can be discussed in bioleaching system.

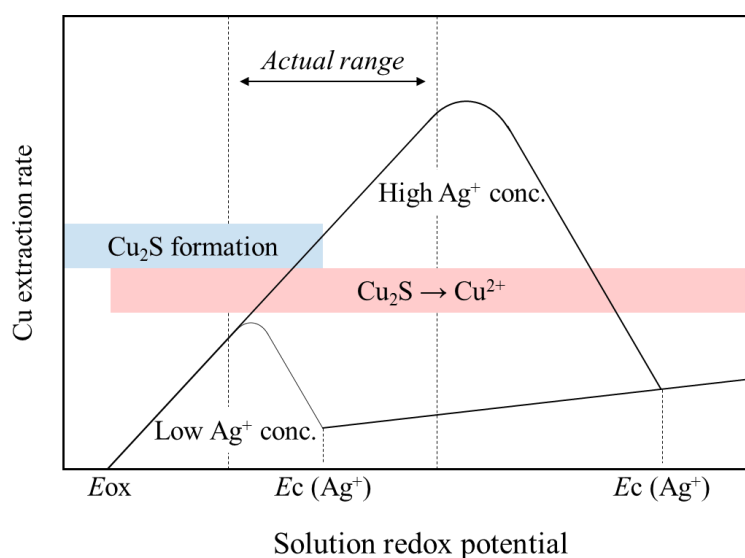


Fig. 7.1 Schematic model of redox dependence of chalcopyrite leaching efficiency in the presence of silver ions.

Solution redox potentials have reported to strongly affect Cu extraction rate in chalcopyrite bio and chemical leaching system by many research groups (Hiro Yoshi et al 2001, 2004, 2008, Third et al 2000, Okamoto et al 2004, Córdoba et al 2008, Vilcáez

et al 2009). Since addition of Ag catalyst to chalcopyrite leaching system results in decrease in solution redox potentials due to accumulation of Fe(II), this chapter was also aimed to investigate the effect of Ag catalyst addition on solution redox potentials and behaviors in solution redox potentials in the presence of Ag catalyst. In this chapter, changes in E_{normal} values were discussed assuming that chalcopyrite dissolution is based on formation of chalcocite as an intermediate.

7.2 Section 1 Oxidative bioleaching of chalcopyrite in the presence of silver sulfate using pure or mixed cultures of moderately thermophiles

7.2.1 Materials and methods

7.2.1.1 Growth of *Acidiplasma* sp. Fv-Ap at extremely acidic pHs

Successive cultivation of *Acidiplasma* sp. Fv-Ap at pH 1.0 was conducted several times. Cells of *Acidiplasma* sp. Fv-Ap were pre-grown, harvested at the late-exponential phase by centrifugation (12,000 g, 10 min at 40°C), washed twice with flesh HBS media (pH 1.0 with H₂SO₄). Microbial cell growth test was conducted in 100 mL Erlenmeyer flasks containing 50 mL HBS media (pH 1.0, 0.9, 0.8, 0.7, and 0.6 with H₂SO₄) supplemented with 0.01% (w/v) yeast extract, 30 mM Fe(II). The initial cell density was set to 1×10^7 cells/mL. All the cultures were maintained at 45°C on an orbital rotary shaker at 140 rpm. Samples were regularly taken to monitor cell density and concentrations of total soluble Fe and Fe(II). All the experiments were carried out in duplicates.

7.2.1.2 Silver catalyzed bioleaching of chalcopyrite using pure culture of *Acidiplasma* sp. Fv-Ap, at extremely acidic pH; 0.7

Successive cultivation of *Acidiplasma* sp. Fv-Ap at pH 0.7 was conducted several times. Cells of *Acidiplasma* sp. Fv-Ap were pre-grown, harvested at the late-exponential phase by centrifugation (12,000 g, 10 min at 40°C), washed twice with flesh HBS media (pH 0.7 with H₂SO₄; containing no Cl ions). All the bioleaching tests were conducted in 500 mL Erlenmeyer flasks containing 200 mL HBS media (pH 0.7 with H₂SO₄; containing no Cl ions) with 0.01% (w/v) yeast extract, 40 mM Fe(II) and 1% (w/v) acid washed-chalcopyrite concentrate. The initial cell densities were set to 1×10^7 cells/mL. Silver catalyst was added in the soluble form of silver sulfate (Ag₂SO₄) to the final concentration of 5 mg/L as Ag(I) at day 10. All the media were maintained at 45°C on an orbital rotary shaker at 140 rpm. Samples were regularly taken to monitor cell density, pH values, solution redox potential values, and concentrations of Fe(II), total soluble Fe and Cu. All the experiments were carried out in duplicates. Residues were analyzed by XRD and observed by SEM.

7.2.1.3 Silver catalyzed bioleaching of chalcopyrite using mixed culture of moderately thermophilic acidophiles, *At. caldus* KU and either *Acidiplasma* sp. Fv-Ap or *Am. ferrooxidans* ICP at initial pH of 2.0

Cells of *At. caldus* KU, *Acidiplasma* sp. Fv-Ap and *Am. ferrooxidans* ICP were pre-grown, harvested at the late-exponential phase by centrifugation (12,000 g, 10 min at 40°C), washed twice with flesh HBS media (pH 1.5 with H₂SO₄; containing no Cl ions). All the bioleaching tests were conducted in 500 mL Erlenmeyer flasks containing 200 mL HBS media (pH 2.0 with H₂SO₄; containing no Cl ions) with 0.01% (w/v) yeast extract, 5 mM Fe(II), 0.5 mg/L Ag(I) (Ag₂SO₄), and 1% (w/v) acid washed-chalcopyrite concentrate. The initial cell densities were set to 2×10^7 cells/mL. All the media were maintained at 45°C on an orbital rotary shaker at 150 rpm. Samples were regularly taken to monitor cell density, pH values, solution redox potential values, and concentrations of Fe(II), total soluble Fe and Cu. All the experiments were carried out in duplicates. Residues were analyzed by XRD and observed by SEM.

7.2.1.4 Silver catalyzed bioleaching of chalcopyrite using mixed culture of moderately thermophilic acidophiles, *At. caldus* KU and *Acidiplasma* sp. Fv-Ap at initial pHs of 1.3 and 1.6

Successive cultivation of *At. caldus* KU and *Acidiplasma* sp. Fv-Ap at pH 1.0 was separately conducted several times. Cells of both strains were pre-grown, harvested at the late-exponential phase by centrifugation (12,000 g, 10 min at 40°C), washed twice with flesh HBS media (pH 1.5 with H₂SO₄; containing no Cl ions). All the bioleaching tests were conducted in 500 mL Erlenmeyer flasks containing 200 mL HBS media (pH 1.3 or 1.6 with H₂SO₄; containing no Cl ions) with 0.01% (w/v) yeast extract, 20 mM Fe(II) and 1% (w/v) acid washed-chalcopyrite concentrate. The initial cell densities were set to 2×10^7 cells/mL. Silver catalyst was added in the soluble form of silver sulfate (Ag₂SO₄) to the final concentration of 5 mg/L as Ag(I) at day 10. All the media were maintained at 45°C on an orbital rotary shaker at 140 rpm. Samples were regularly taken to monitor cell density, pH values, solution redox potential values, and concentrations of Fe(II), total soluble Fe and Cu. All the experiments were carried out in duplicates. Residues were analyzed by XRD and observed by SEM.

7.2.2 Results and discussion

7.2.2.1 Growth of *Acidiplasma* sp. Fv-Ap at extremely acidic pHs

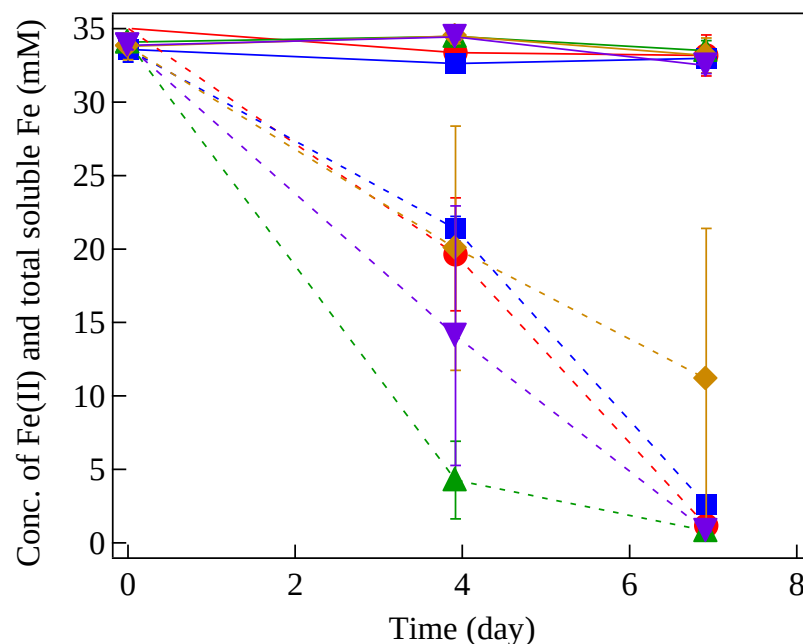


Fig. 7.2 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) in inoculated cultures of *Acidiplasma* sp. Fv-Ap at initial pHs of 1.0 (●), 0.9 (■), 0.8 (▲), 0.7 (◆), and 0.6 (▼), respectively.

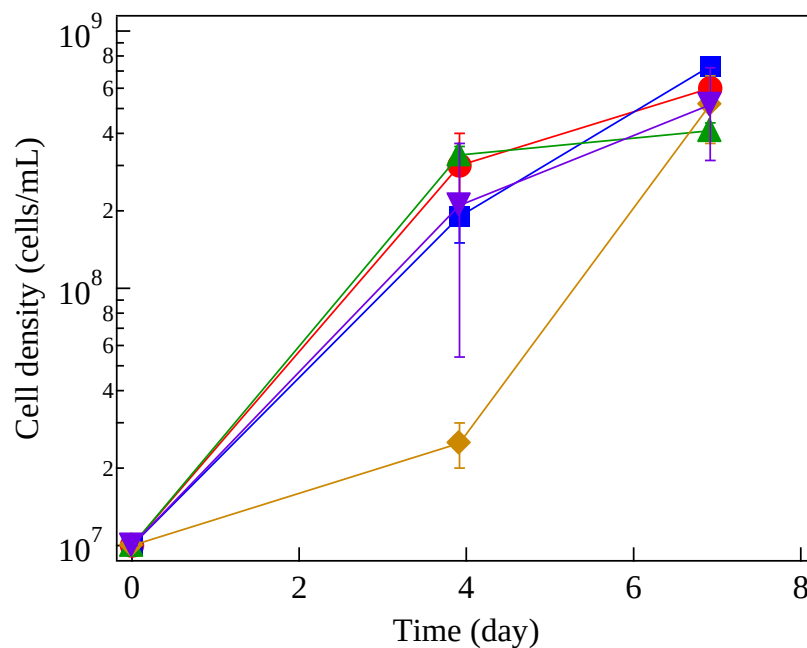


Fig. 7.3 Changes in the cell density in inoculated cultures of *Acidiplasma* sp. Fv-Ap at initial pHs of 1.0 (●), 0.9 (■), 0.8 (▲), 0.7 (◆), and 0.6 (▼).

Acidiplasma sp. Fv-Ap is moderately thermophilic, heterotrophic, and acidophilic Fe(II)-oxidizing archaeon. Microbial cell growth of *Acidiplasma* sp. Fv-Ap was found even at extremely acidic pHs (initial); 1.0, 0.9, 0.8, 0.7, and 0.6. Lag in cell growth at different initial pHs was not observed, and furthermore, final cell density at day 7 in all inoculated cultures was almost similar; 4×10^8 to 7×10^8 cells/mL. Microbial Fe(II) oxidation (coupled to cell growth) was also observed in all the inoculated cultures of *Acidiplasma* sp. Fv-Ap: 92-98% of Fe(II) (initially 30 mM added) were oxidized within 7 days by Fe(II)-oxidizing ability of *Acidiplasma* sp. Fv-Ap in all the inoculated cultures at initial pHs tested (except for a pair of duplicates at initial pH of 0.7). No ferric precipitation occurred even after almost complete oxidation of Fe(II), due to extremely acidic pHs.

Cell growth at pH 0.6 implies that *Acidiplasma* sp. Fv-Ap is likely extremely acidophilic archaeon. The fact motivated us to use the archaeon to chalcopyrite bioleaching at extremely acidic pHs.

7.2.2.2 Chalcopyrite bioleaching test in the presence of silver sulfate using pure culture of *Acidiplasma* sp. Fv-Ap, at extremely acidic pH; 0.7

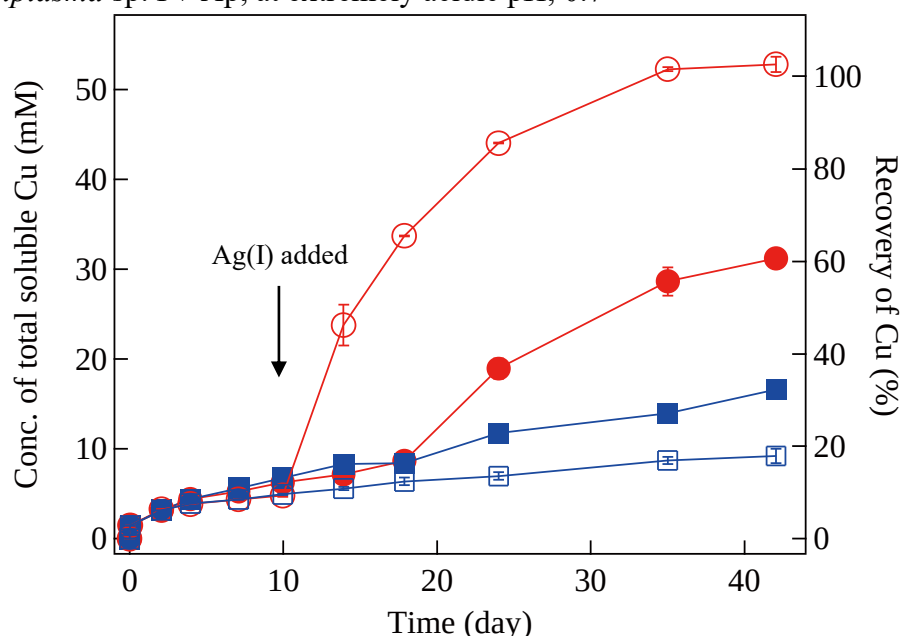


Fig. 7.4 Changes in the concentrations of total soluble Cu leached with (●, ○) and without (■, □) the addition of Ag(I) at day 10, at initial pH of 0.7. Open and filled symbols indicate inoculated cultures and sterile controls, respectively.

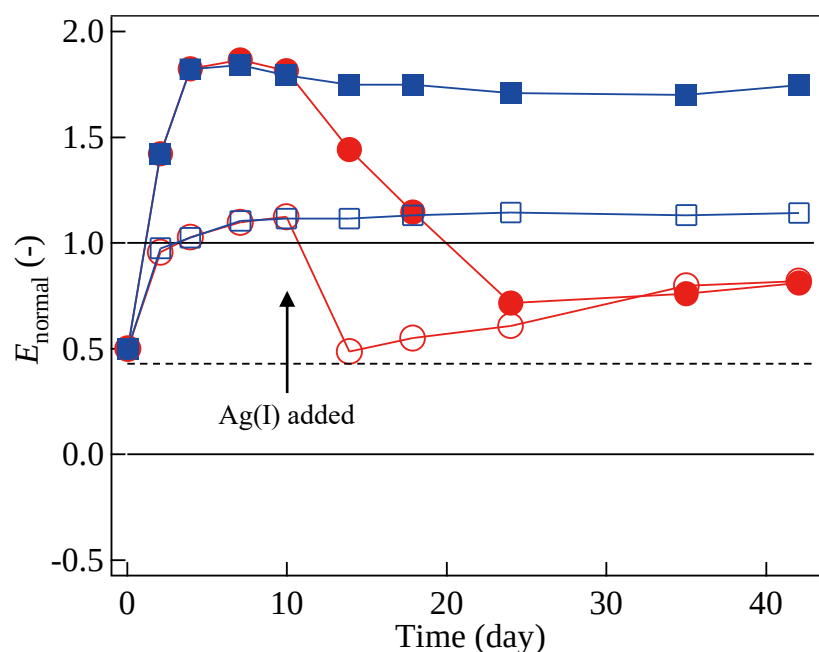


Fig. 7.5 Changes in the E_{normal} values with (●, ○) and without (■, □) the addition of Ag(I) at day 10, at initial pH of 0.7. Open and filled symbols indicate inoculated cultures and sterile controls, respectively. Dotted line; $E_{\text{normal}}=0.43$.

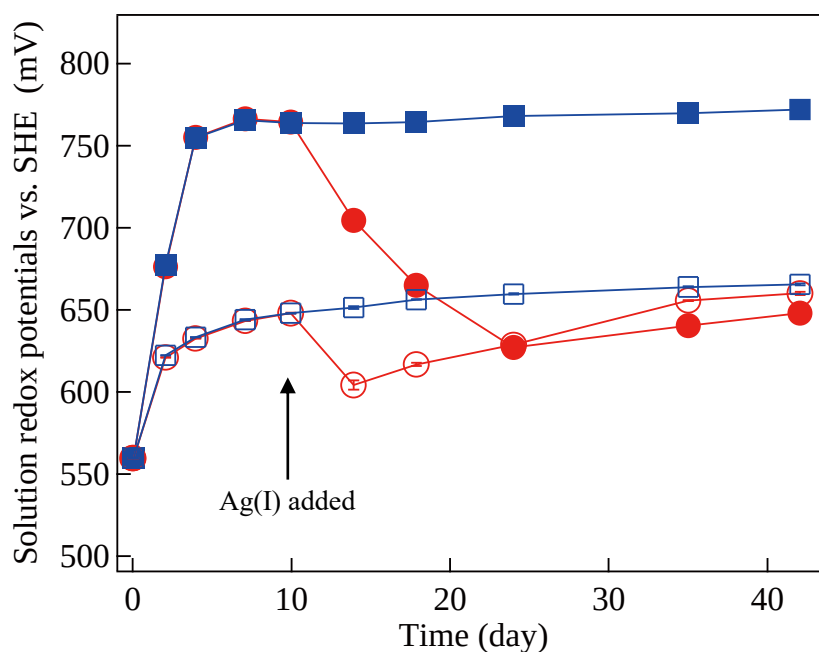


Fig. 7.6 Changes in the solution redox potentials with (●, ○) and without (■, □) the addition of Ag(I) at day 10, at initial pH of 0.7. Open and filled symbols indicate inoculated cultures and sterile controls, respectively.

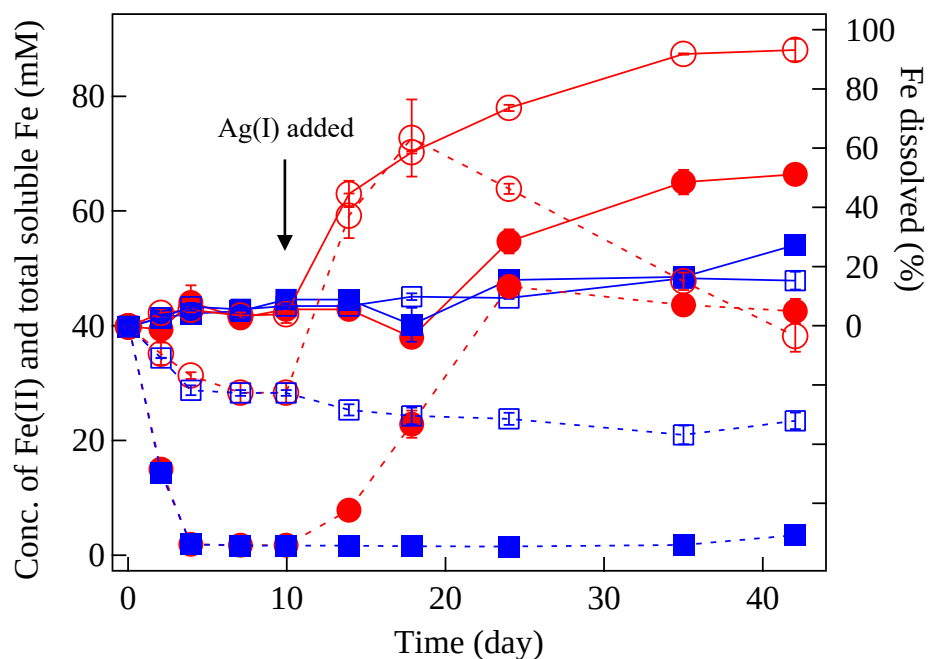


Fig. 7.7 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) leached with (●, ○) and without (■, □) the addition of Ag(I) at day 10, at initial pH of 0.7. Open and filled symbols indicate inoculated cultures and sterile controls, respectively.

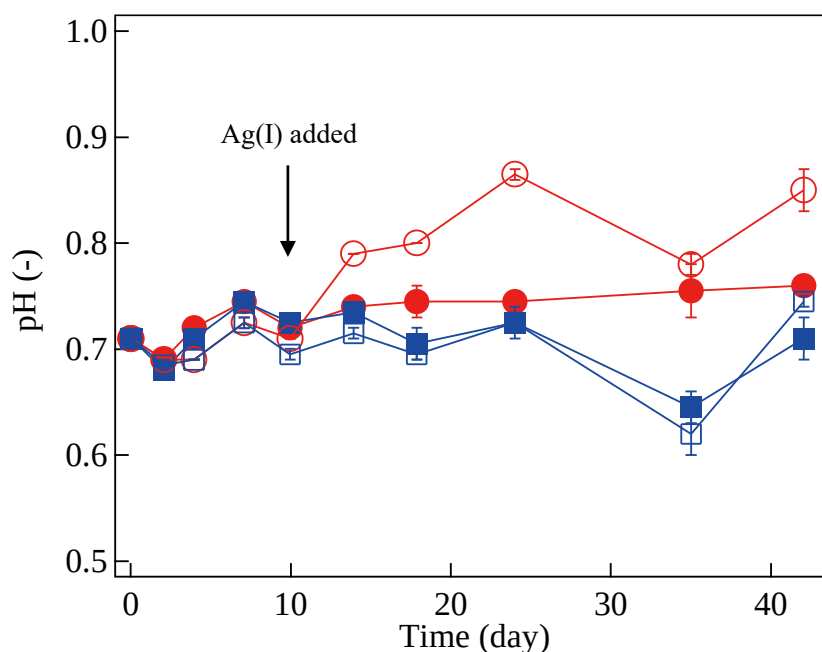


Fig. 7.8 Changes in the pH values with (●, ○) and without (■, □) the addition of Ag(I) at day 10, at initial pH of 0.7. Open and filled symbols indicate inoculated cultures and sterile controls, respectively.

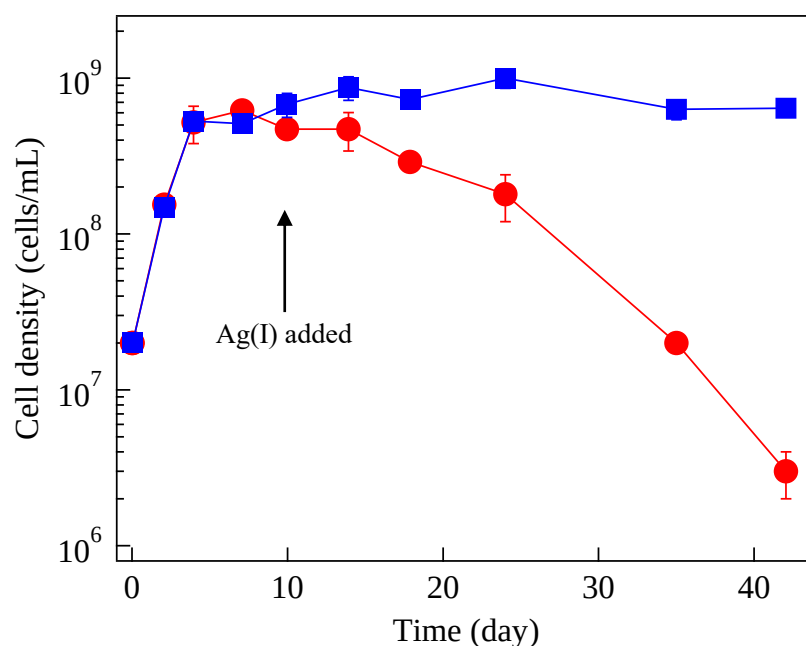


Fig. 7.9 Changes in the cell density with (●) and without (■) the addition of Ag(I) at day 10, at initial pH of 0.7.

In this experiment, effect of the addition of Ag(I) ions as a catalyst to the chalcopyrite bioleaching system at pH 0.7 was investigated. Before the addition of Ag(I) to the bioleaching system at day 10, the E_{normal} values increased over 1.8 (corresponding to 750 mV as solution redox potentials) owing to the microbial Fe(II)-oxidizing activity of *Acidiplasma* sp. Fv-Ap, while that in sterile controls were increased to approximately 1.1 (corresponding to 650 mV) due to weaker Fe(II) oxidation phenomenon by dissolved oxygen. Active cell growth of *Acidiplasma* sp. Fv-Ap was also observed within 4 days and cell density was maintained the level until day 10. After the addition of 5 mg/L Ag(I) at day 10, Cu extraction in sterile controls was immediately enhanced and final Cu recovery at day 42 reached 100%, while the final Cu recovery without the addition of Ag(I) at the end of the experiment was below 20% (due to higher E_{normal} values over 1 throughout the experiment). The immediate enhancement of Cu extraction rate was mainly caused by two different mechanisms. One is the catalytic activity of Ag(I) as is shown in Eq. 7.3-7.5. The other factor for the immediate increase in Cu concentration after the addition of Ag(I) in sterile controls, is the E_{normal} values: The values decreased down to 0.5 ($0 < E_{\text{normal}} < 1$) right after the addition of Ag(I), caused by (1) accumulation of Fe(II) and (2) a lack of Fe(III)

available to Ag_2S oxidation, as shown in Eq. 7.5 and 7.6. Furthermore, in sterile controls with the addition of Ag(I) , gradual increase in solution redox potentials, as well as the E_{normal} values, after day 14 induced gradual declining in Cu dissolution.

Although significant improvement of Cu extraction rate right after the addition of Ag(I) in sterile controls was confirmed, response of Cu extraction rate to the addition of Ag(I) in inoculated cultures delayed: Enhancement effect of Ag(I) addition at day 10 to Cu extraction rate in inoculated cultures was found at day 18-24. This may be also due to the changes in the E_{normal} values. Before the addition of Ag(I) at day 10, the E_{normal} values in inoculated cultures maintained around 1.8 ($1 < E_{\text{normal}}$), and after the addition of Ag(I) , the values decreased to 1.1 at day 18, subsequently to 0.7 at day 24. These results indicate that Cu extraction was readily boosted right after the E_{normal} values falls within the range ($0 < E_{\text{normal}} < 1$). Moreover, Cu extraction rate at day 18-24 in inoculated cultures was less effective than that at day 10-14 in sterile controls, caused by that the difference in the E_{normal} levels: The E_{normal} value in the former (at day 18) was higher than that in the latter (at day 10). In addition, the reason for that Cu extraction rate at day 24-42 between inoculated cultures and sterile controls was almost similar each other, was likely almost similar behavior in the E_{normal} values in both cases.

In this experiment, when Ag(I) was added at day 10, sterile controls marked higher Cu recovery (100%) at the end of the experiment compared with inoculated cultures (60%). The most likely reason for the result is microbial activity of Fe(II) oxidation in inoculated cultures: Fe(II) -oxidizing activity of *Acidiplasma* sp. Fv-Ap seemed to inhibit the E_{normal} values to decrease down to the midl level of the range ($0 < E_{\text{normal}} < 1$), although decrease in cell density was observed after the addition of Ag(I) at day 10. Overall, in order to show positive impact of Ag(I) addition to the chalcopyrite bioleaching, the balance between the amount of Ag_2S added and microbial Fe(II) -oxidizing activity, was indicated to be important.

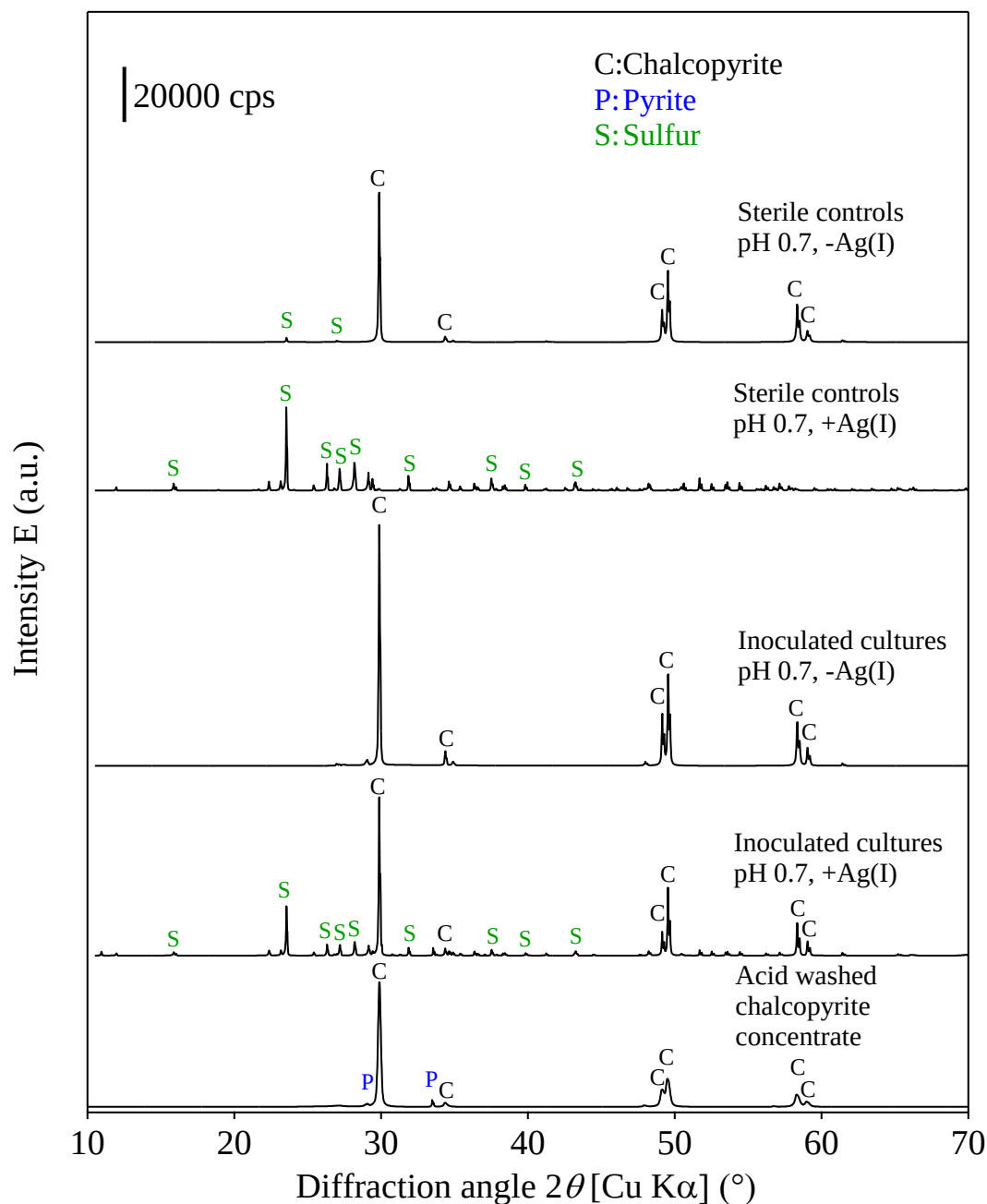


Fig. 7.10 X-ray diffraction patterns of original chalcopyrite concentrate, residual mineral in inoculated cultures and sterile controls with and without the addition of Ag(I), respectively, from the bottom to the top. “C”, “P”, and “S” indicate the peak patterns corresponding to chalcopyrite, pyrite, and sulfur, respectively.

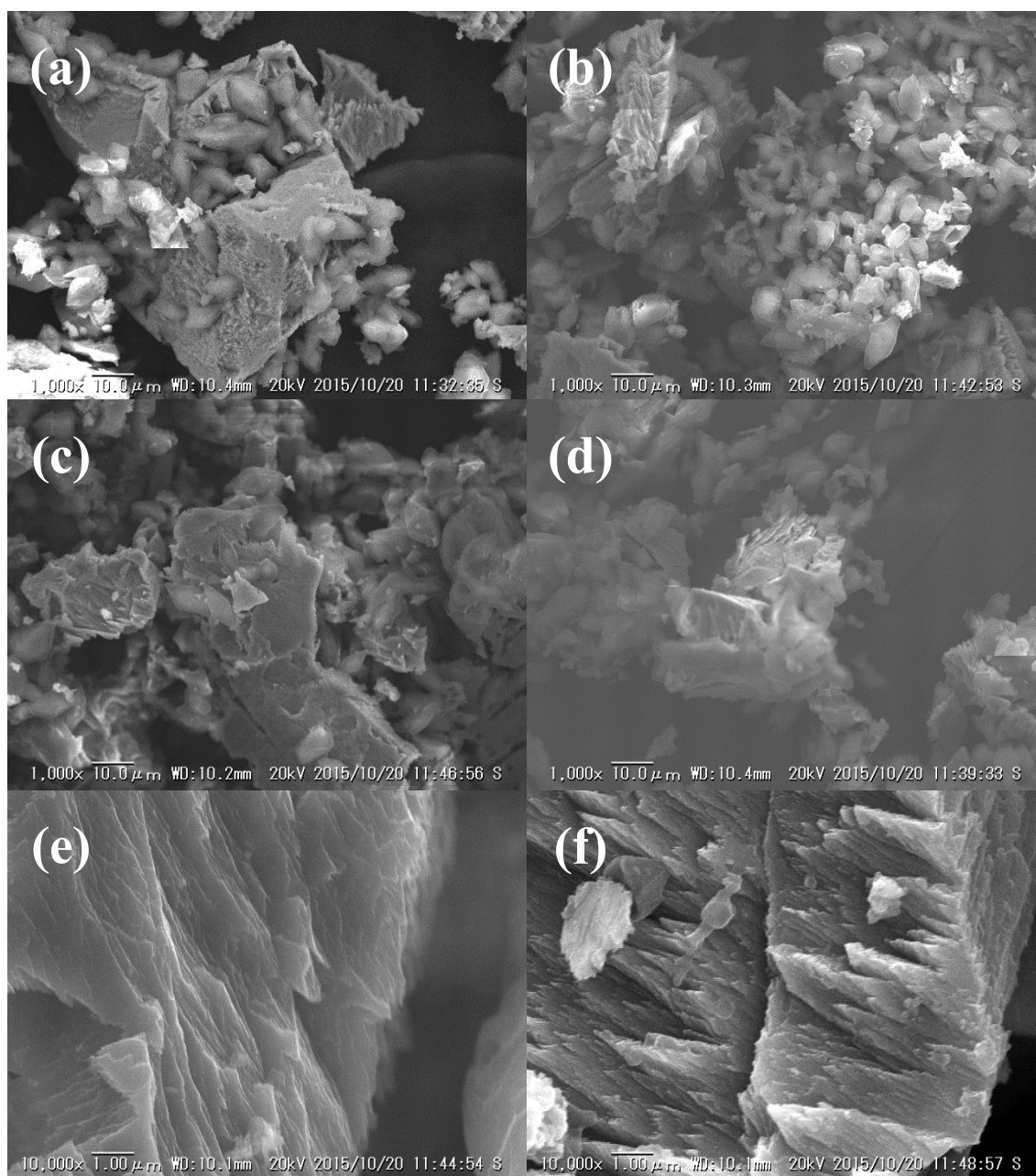


Fig. 7.11 SEM images of bioleached residues in inoculated cultures of *Acidiplasma* sp. Fv-Ap with the addition of Ag(I) at day 10, at initial pH of 0.7. (a), (b), (c), and (d) indicate the images at magnification of 1,000×. (e) and (f) indicate the images at magnification of 10,000×.

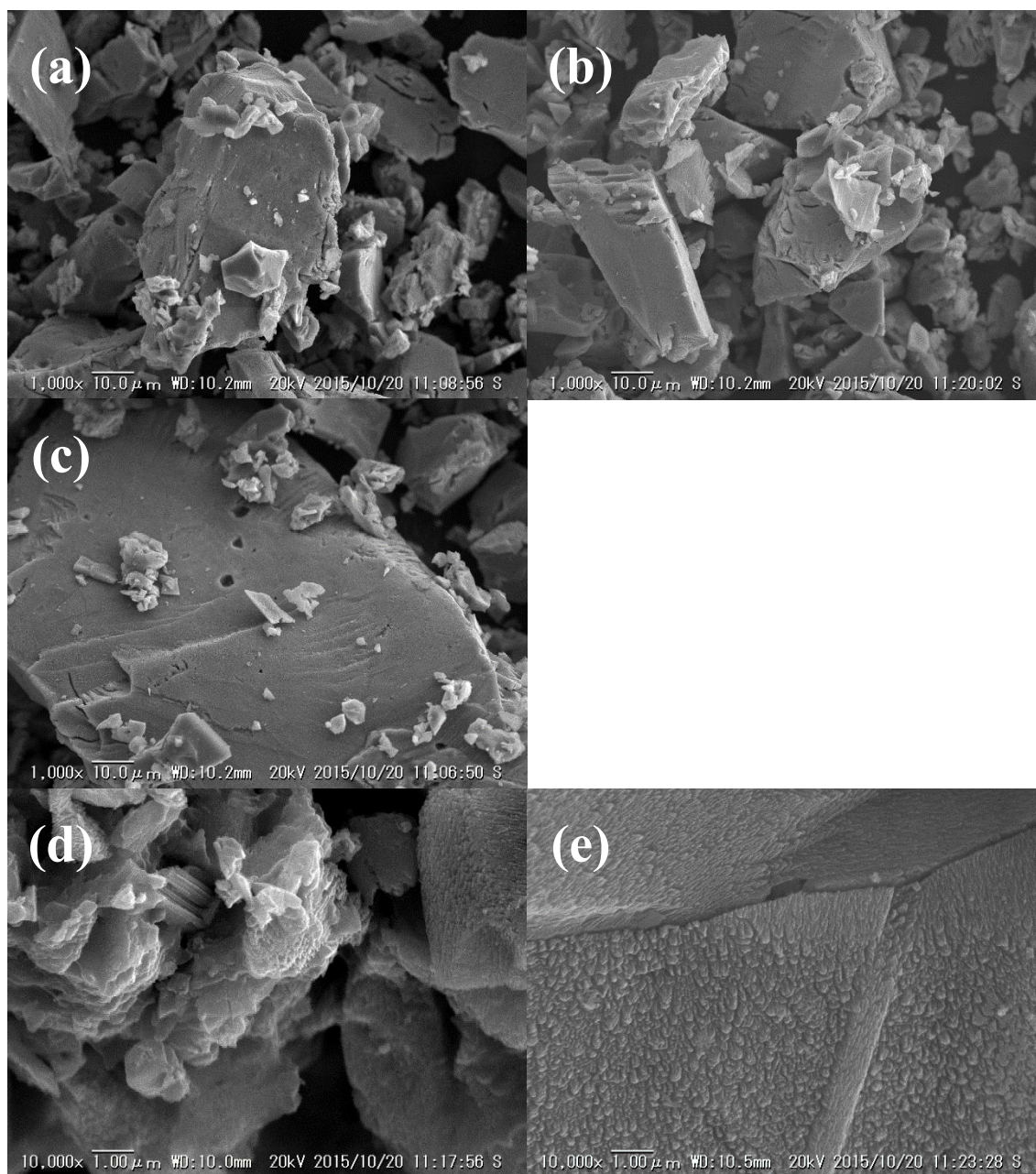


Fig. 7.12 SEM images of bioleached residues in inoculated cultures of *Acidiplasma* sp. Fv-Ap without the addition of Ag(I) at day 10, at initial pH of 0.7. (a), (b), and (c) indicate the images at magnification of 1,000×. (d) and (e) indicate the images at magnification of 10,000×.

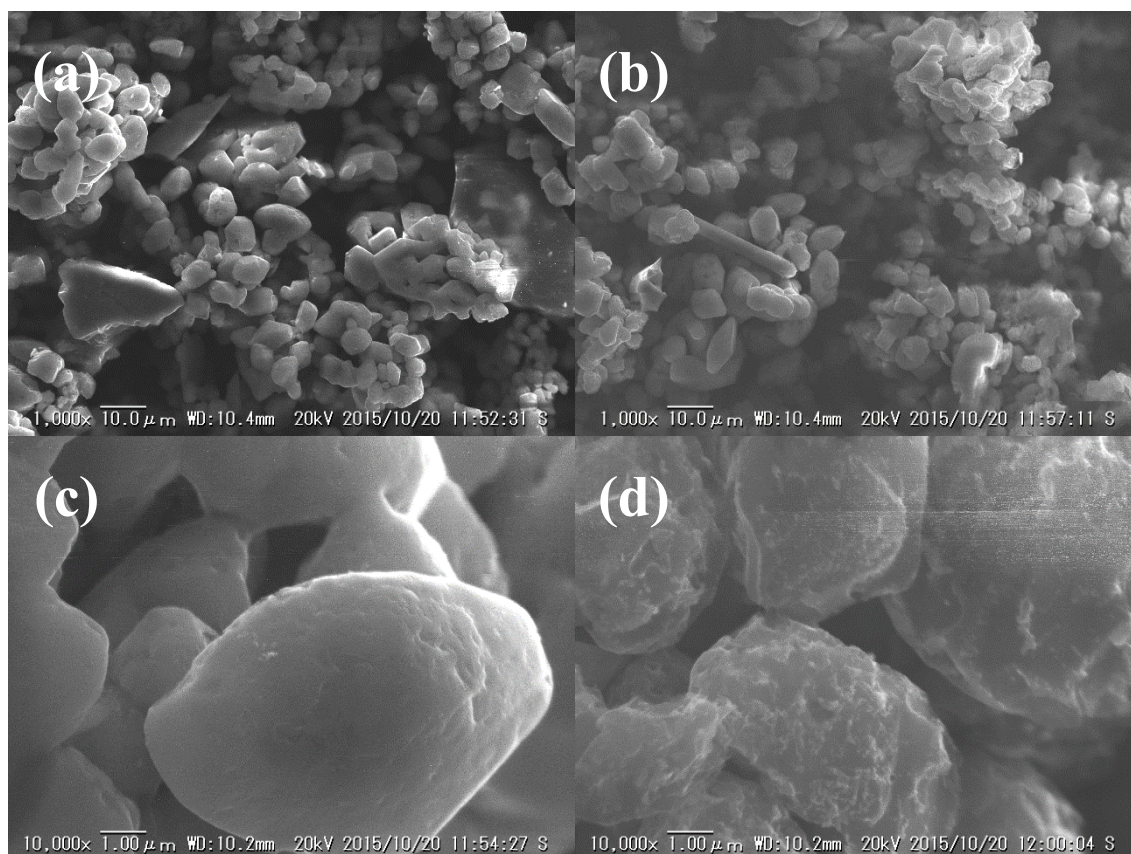


Fig. 7.13 SEM images of bioleached residues in sterile controls with the addition of Ag(I) at day 10, at initial pH of 0.7. (a) and (b) indicate the images at magnification of 1,000 $\times$. (c) and (d) indicate the images at magnification of 10,000 $\times$.

According to the XRD analysis and SEM observation, when Ag(I) was not added, the presence of only small amount of elemental sulfur was confirmed in sterile controls, and XRD peak pattern corresponding to elemental sulfur was absent in inoculated cultures, maybe caused by relatively low dissolution rate of chalcopyrite throughout the experiment. Since Cu recoveries at day 42 in inoculated cultures and sterile controls were 60 and 100%, respectively, formation of elemental sulfur was shown with the larger amount in the latter than that in the former. Due to the extremely acidic pH of the bioleaching conditions, formation of ferric precipitates such as jarosite was not observed at day 42 in both inoculated cultures and sterile controls.

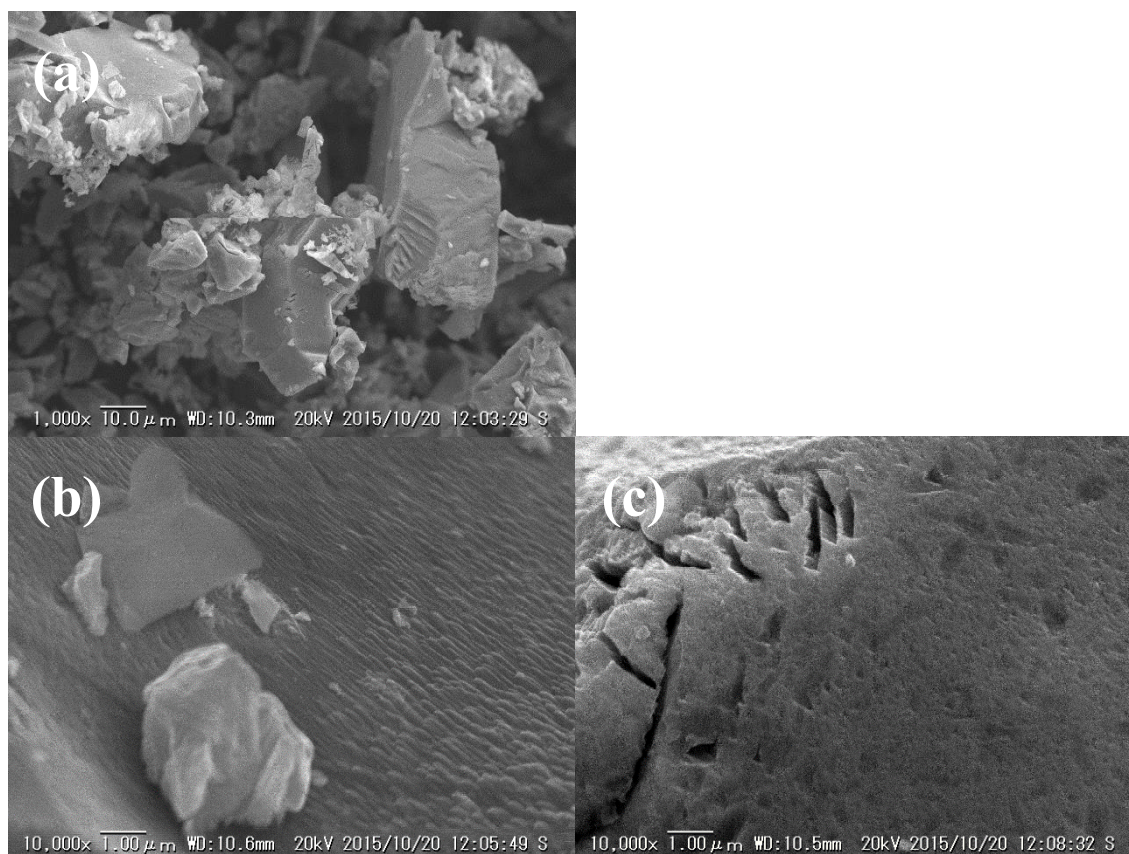


Fig. 7.14 SEM images of bioleached residues in sterile controls without the addition of Ag(I) at day 10, at initial pH of 0.7. (a) indicates the images at magnification of 1,000×. (b) and (c) indicate the images at magnification of 10,000×.

7.2.2.3 Chalcopyrite bioleaching test in the presence of silver sulfate using mixed culture of moderately thermophilic acidophiles, *At. caldus* KU and either *Acidiplasma* sp. Fv-Ap or *Am. ferrooxidans* ICP at initial pH of 2.0

In all mixed cultures regardless of the Ag(I) addition, since (1) active cell growth was observed and maintained at the relatively high levels, (2) gradual decrease in pH values was observed, and (3) solution redox potentials were maintained at the level of

750-800 mV (corresponding to the E_{normal} values of 1.3-2.0), microbial oxidation of both Fe(II) and S^0 was implied not to be inhibited by the presence of Ag(I) or Ag_2S throughout the experiment. Regardless of the addition of Ag(I), Cu recovery in all mixed cultures tested reached 49-56% at day 70, while that in sterile controls was 17%. Unexpectedly, effect of the addition of Ag(I) to bioleaching systems on Cu recovery was not shown, which might have been caused by the fact that the initial concentration of Ag(I); 0.5 mg/L, was too low to show its catalytic capability for chalcopyrite dissolution. In order to evaluate the effect of the addition of Ag(I) to bioleaching systems on Cu recovery, Ag(I) concentration was set to 5 mg/L and the addition was carried out at day 10 to minimize its negative effect on microbial growth in the next experiments.

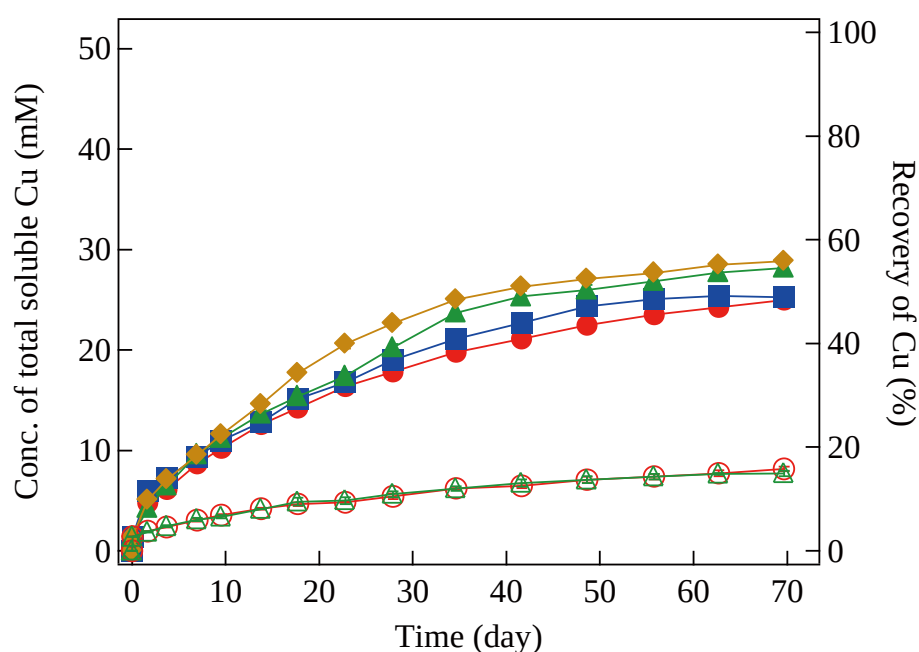


Fig. 7.15 Changes in the concentrations of total soluble Cu leached in mixed cultures of *At. caldus* KU and either *Acidiplasma* sp. Fv-Ap (●, ▲) or *Am. ferrooxidans* ICP (■, ◆) and sterile controls (○, △). Ag(I) was initially added to mixed cultures (▲, ◆) and sterile control (△).

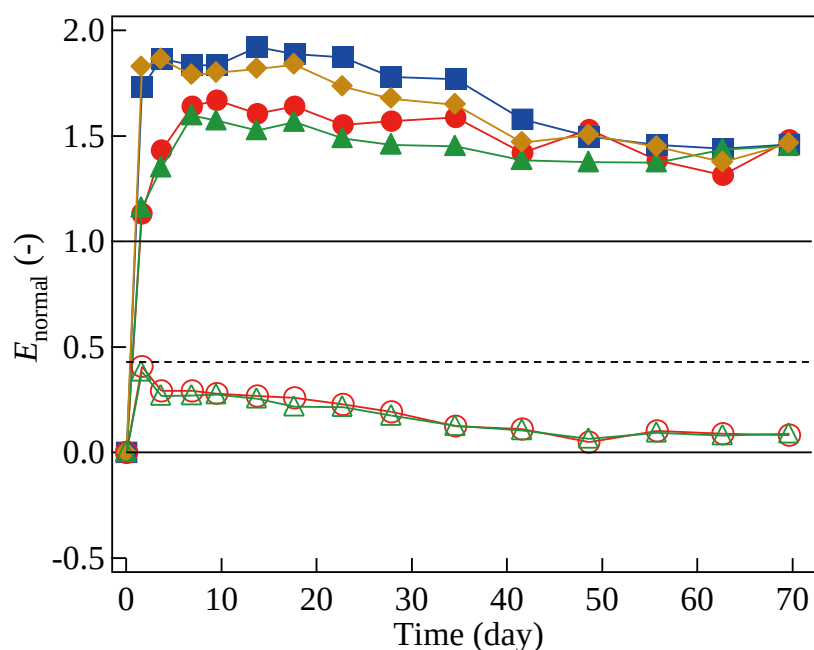


Fig. 7.16 Changes in the E_{normal} values in mixed cultures of *At. caldus* KU and either *Acidiplasma* sp. Fv-Ap (●, ▲) or *Am. ferrooxidans* ICP (■, ◆) and sterile controls (○, △). Ag(I) was initially added to mixed cultures (▲, ◆) and sterile control (△). Dotted line; $E_{\text{normal}}=0.43$.

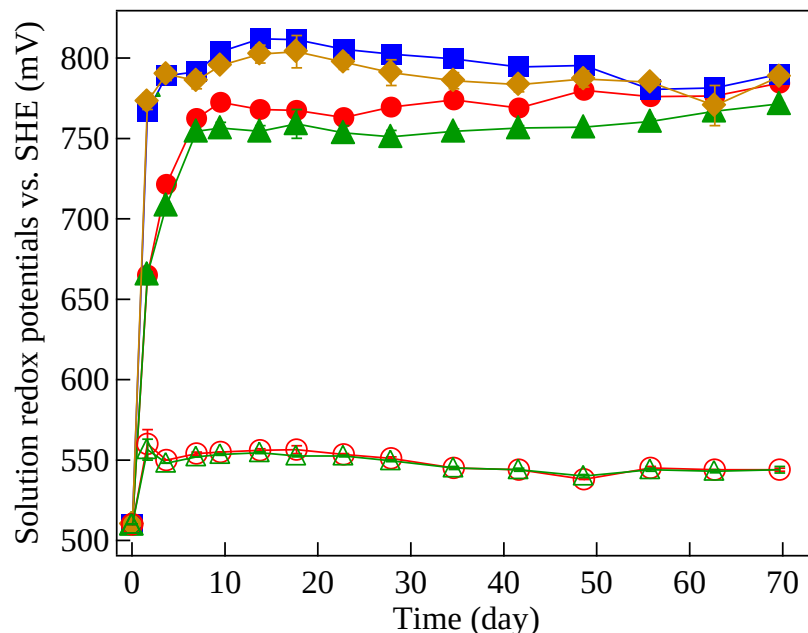


Fig. 7.17 Changes in the solution redox potentials in mixed cultures of *At. caldus* KU and either *Acidiplasma* sp. Fv-Ap (●, ▲) or *Am. ferrooxidans* ICP (■, ◆) and sterile controls (○, △). Ag(I) was initially added to mixed cultures (▲, ◆) and sterile control (△).

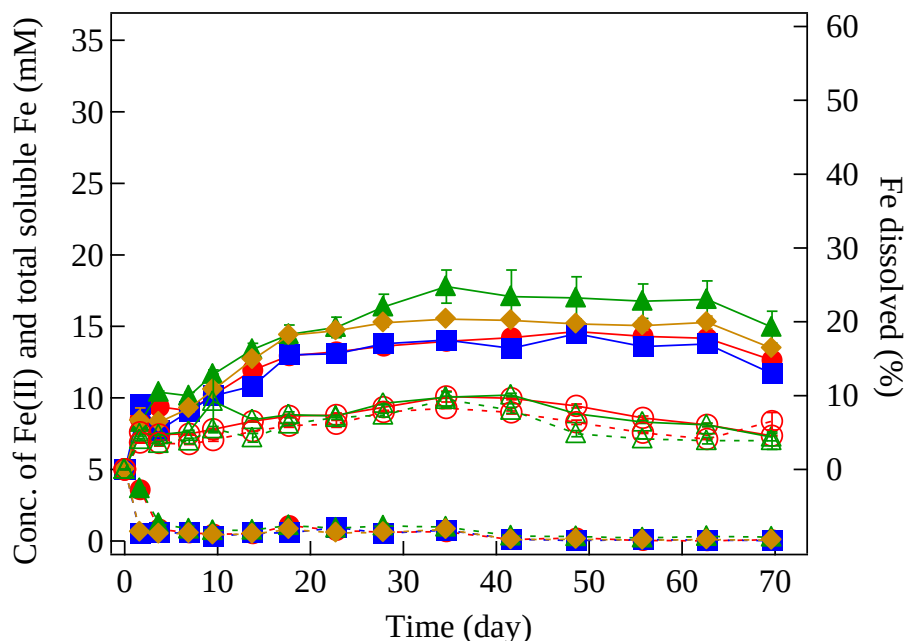


Fig. 7.18 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) leached in mixed cultures of *At. caldus* KU and either *Acidiplasma* sp. Fv-Ap (●, ▲) or *Am. ferrooxidans* ICP (■, ◆) and sterile controls (○, △). Ag(I) was initially added to mixed cultures (▲, ◆) and sterile control (△), respectively.

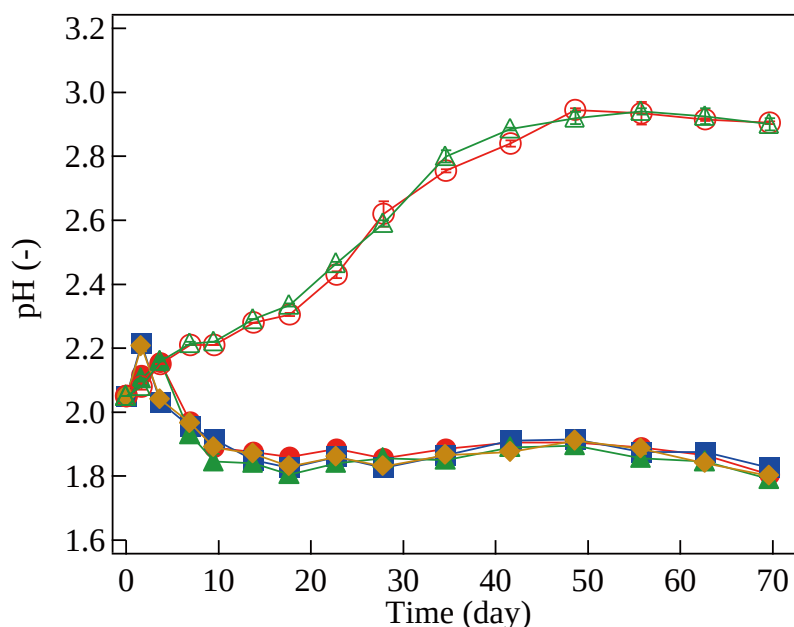


Fig. 7.19 Changes in the pH values in mixed cultures of *At. caldus* KU and either *Acidiplasma* sp. Fv-Ap (●, ▲) or *Am. ferrooxidans* ICP (■, ◆) and sterile controls (○, △). Ag(I) was initially added to mixed cultures (▲, ◆) and sterile control (△).

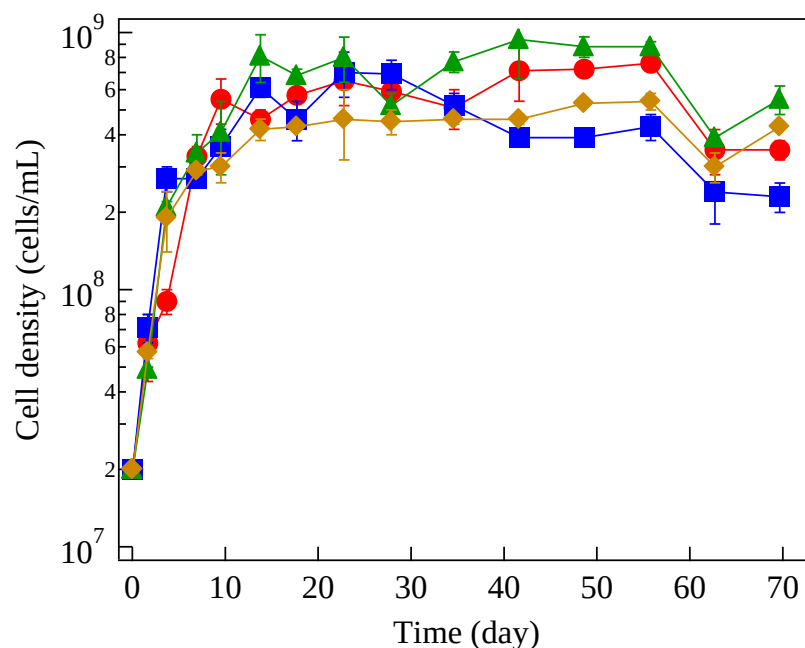


Fig. 7.20 Changes in the cell density in mixed cultures of *At. caldus* KU and either *Acidiplasma* sp. Fv-Ap (●, ▲) or *Am. ferrooxidans* ICP (■, ◆). Ag(I) was initially added to mixed cultures (▲, ◆).

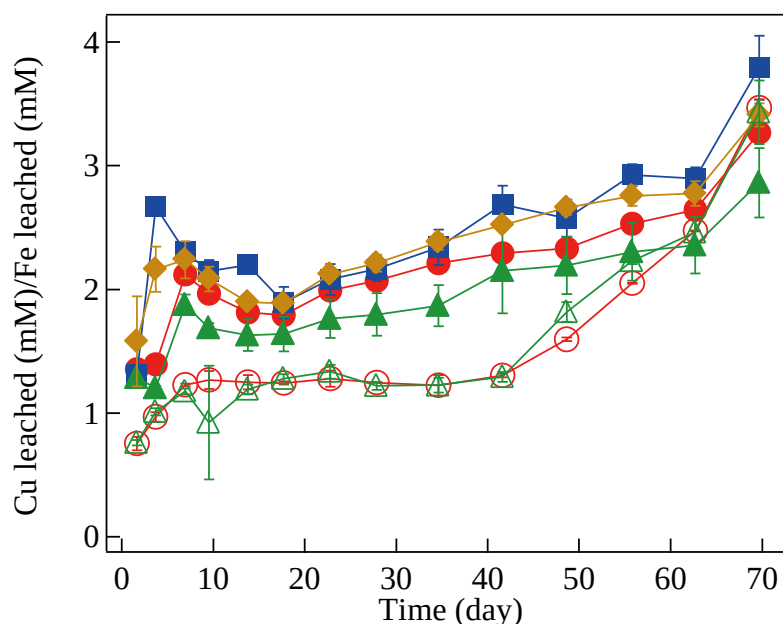


Fig. 7.21 Changes in the molar ratio between Cu and Fe leached in mixed cultures of *At. caldus* KU and either *Acidiplasma* sp. Fv-Ap (●, ▲) or *Am. ferrooxidans* ICP (■, ◆) and sterile controls (○, △). Ag(I) was initially added to mixed cultures (▲, ◆) and sterile control (△).

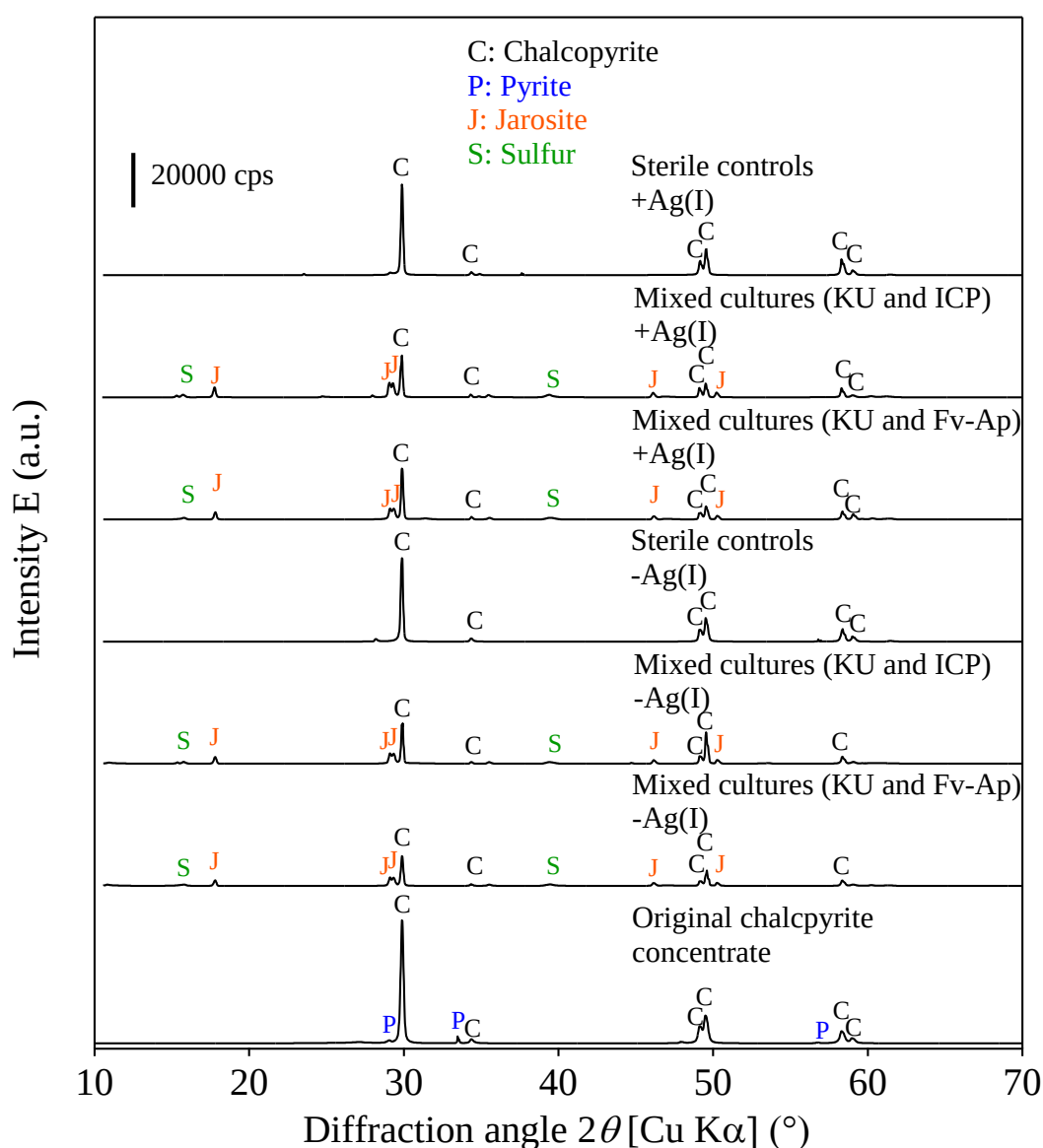


Fig. 7.22 X-ray diffraction patterns of original chalcopyrite concentrate, residual mineral in mixed cultures of *At. caldus* KU mixed with and either *Acidiplasma* sp. Fv-Ap or *Am. ferrooxidans* ICP and sterile controls without and with the addition of Ag(I), respectively, from the bottom to the top. “C”, “P”, “S”, and “J” indicate the peak patterns corresponding to chalcopyrite, pyrite, sulfur, and potassium jarosite, respectively.

7.2.2.4 Chalcopyrite bioleaching test in the presence of silver sulfate using mixed culture of moderately thermophilic acidophiles, *At. caldus* KU and *Acidiplasma* sp. Fv-Ap at initial pHs of 1.3 and 1.6

In this experiments. silver sulfate in the form of soluble Ag(I) (5 mg/L) was added as Ag catalyst to the mixed cultures of *At. caldus* KU and *Acidiplasma* sp. Fv-Ap at day 10 when the solution redox potential values reached over 800 mV (based on the Fe(II) oxidation to Fe(III) by the Fe(II)-oxidizing archaeon, *Acidiplasma* sp. Fv-Ap) in order to maintain high solution redox potentials to promote oxidation of chalcopyrite as Eq. 7.8;



Regardless of the initial pHs of the chalcopyrite bioleaching test, active cell growth within the first 2 days in all mixed cultures corresponded to fast Fe(II) oxidation via microbial activity of *Acidiplasma* sp. Fv-Ap, which led to an immediate rise in the the E_{normal} values to around 2.0 (corresponding to nearly 800 mV as solution redox potentials). It has been reported that concentration of soluble Ag(I) in the leachate is limited even though silver catalyst is added as soluble Ag_2SO_4 and AgCl (< 0.1 mg/L) (Ahonen et al 1990). However, cell density gradually declined after the addition of Ag(I) at day 10 in this study, while no decrease in cell density was observed throughout the experiment in the absence of Ag(I).

Effect of Ag(I) addition on chalcopyrite bioleaching at initial pH of 1.3

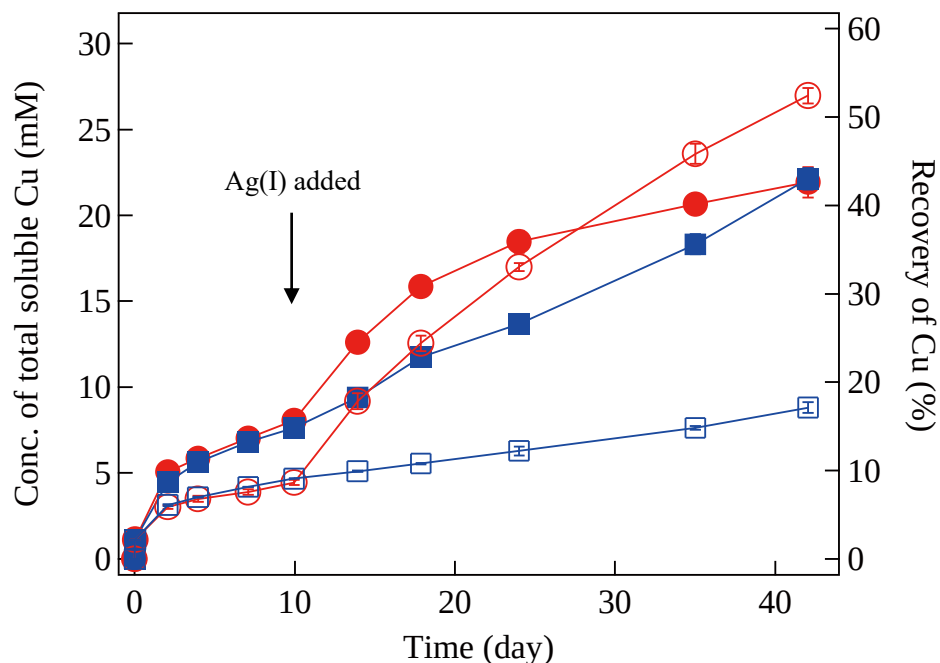


Fig. 7.23 Changes in the concentrations of total soluble Cu leached with (●, ○) and without (■, □) the addition of Ag(I) at day 10, at initial pH of 1.3. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

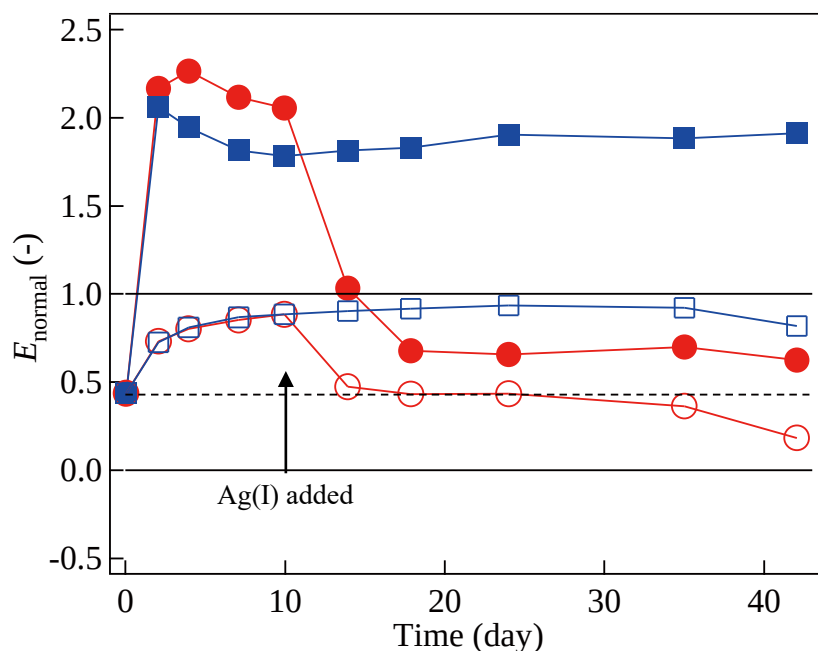


Fig. 7.24 Changes in the E_{normal} values with (●, ○) and without (■, □) the addition of Ag(I) at day 10, at initial pH of 1.3. Open and filled symbols indicate mixed cultures and sterile controls, respectively. Dotted line; $E_{normal}=0.43$.

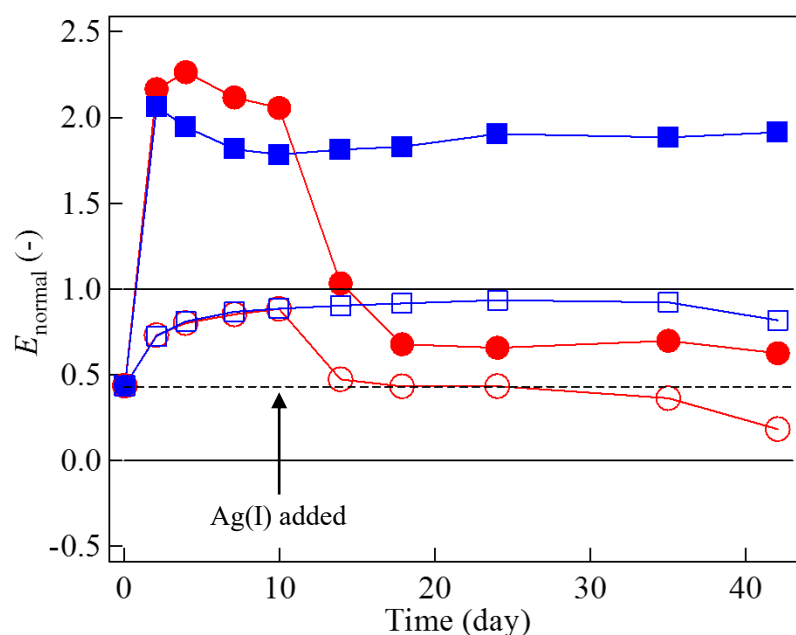


Fig. 7.25 Changes in the solution redox potentials with (●, ○) and without (■, □) the addition of Ag(I) at day 10, at initial pH of 1.3. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

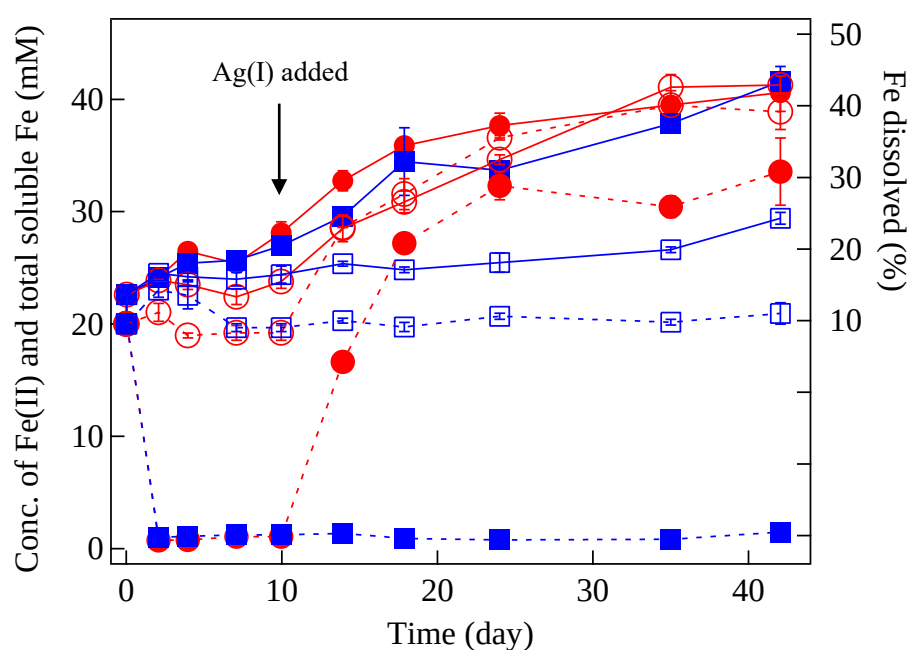


Fig. 7.26 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) leached with (●, ○) and without (■, □) the addition of Ag(I) at day 10, at initial pH of 1.3. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

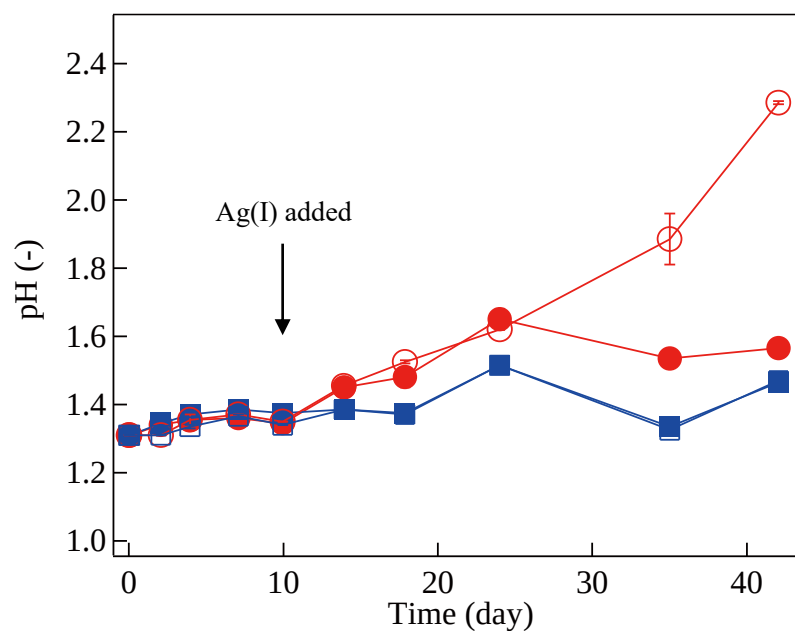


Fig. 7.27 Changes in the pH values with (●, ○) and without (■, □) the addition of Ag(I) at day 10, at initial pH of 1.3. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

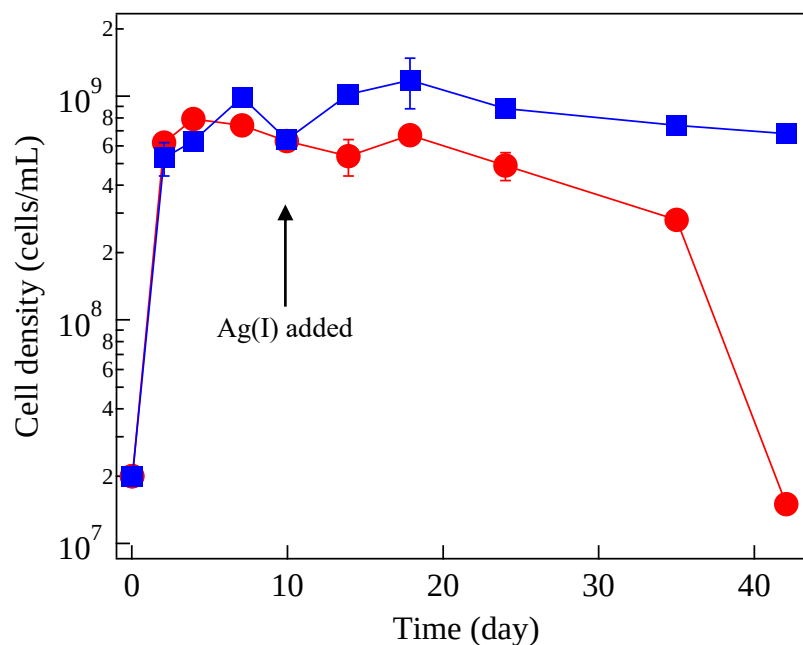


Fig. 7.28 Changes in the cell density with (●) and without (■) the addition of Ag(I) at day 10, at initial pH of 1.3.

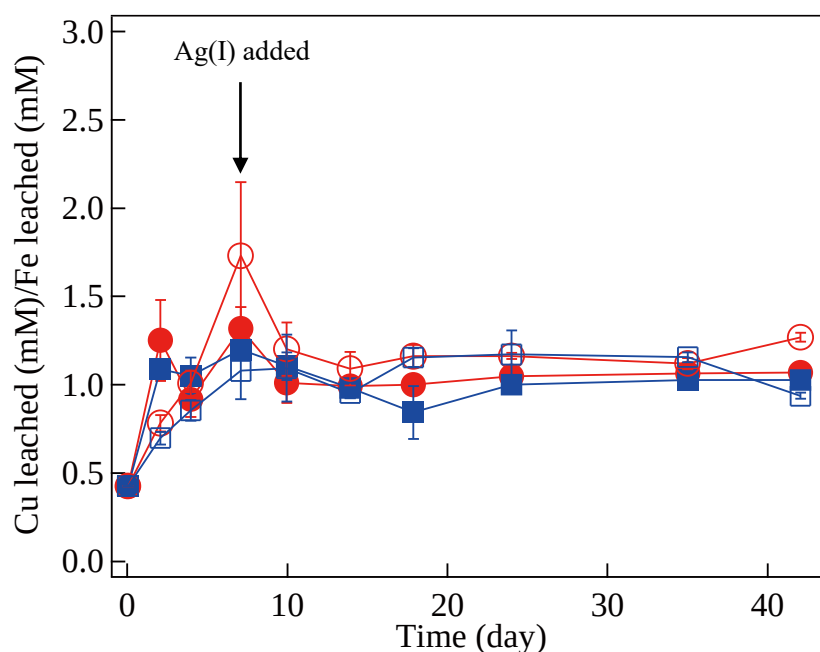


Fig. 7.29 Changes in the molar ratio between Cu and Fe leached with (●, ○) and without (■, □) the addition of Ag(I) at day 10, at initial pH of 1.3. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

(i) Cu recovery in the absence of Ag(I) at initial pH of 1.3:

Cu recovery at day 42 was apparently improved by microbial activities (mixed cultures; 43%, sterile controls; 18%). Nevertheless, pH values of both mixed and sterile controls behaved similarly throughout the experiment, and finally reached to pH 1.47. This indicates that *At. caldus* KU was responsible for S^0 oxidation to produce sulfuric acid, even though *Acidiplasma* sp. Fv-Ap was the dominant species in mixed cultures, based on morphological observation under the microscope. High E_{normal} values around 2.0 (corresponding to solution redox potentials of around 800 mV) was maintained in mixed cultures throughout the experiment since bioleaching system was active.

(ii) Cu recovery in the presence of Ag(I) at initial pH of 1.3:

Addition of Ag(I) to the cultures at day 10 caused a rapid increase in Fe(II) concentration, followed by a rapid drop in solution redox potentials, especially in mixed cultures (from 810 to 620 mV), supposedly based on the Eq. 7.5 and 7.6. Improvement in Cu extraction by the addition of Ag(I) was effectively observed only

in sterile controls (improved from 18 to 52%), while mixed cultures showed 43% Cu recovery at day 42 regardless of the addition of Ag(I), even though Cu recovery was temporally accelerated right after the addition of Ag(I) at day 10. One of possible reasons for this observation might be the formation of secondary layers deposited on the chalcopyrite surface. Córdoba et al (2009) reported the leaching process of chalcopyrite being greatly affected by the formation of jarosite ($\text{MFe}_3(\text{OH})_6(\text{SO}_4)_2$, where $\text{M} = \text{K}^+, \text{Na}^+, \text{NH}_4^+, \text{Ag}^+, \text{or } \text{H}_3\text{O}^+$) and S^0 on the mineral surface. However, the observed ratio Cu leached /Fe leached in mixed cultures was around 1.0 throughout the experiment, showing the theoretical molar ratio during the chalcopyrite leaching, indicating the absence of ferric precipitates formation. As for S^0 , it was reported to accumulate in a large quantity on chalcopyrite surface especially at solution redox potential > 700 mV (López-Juárez et al 2006). Since solution redox potentials in mixed cultures were over 810 mV before the addition of Ag(I) at day 10, S^0 may have passivated the chalcopyrite surface, which was then no longer effectively leached through Ag(I) catalysis. The other possible reason may be the changes in the E_{normal} values: Higher Cu extraction rate in sterile controls was induced by the E_{normal} values to be middle of the range ($0 < E_{\text{normal}} < 1$), the E_{normal} values in sterile controls were maintained at around 0.75, resulting in less effective Cu recovery at day 42.

Comparing the pH profiles of experiments (i) and (ii), sulfuric acid producing activity of *At. caldus* KU can be elucidated: In the presence of Ag(I), 15.8 mM H^+ was produced by *At. caldus* KU, compared with 23.3 mM in the absence of Ag(I). This indicates that activity of *At. caldus* KU during bioleaching was inhibited by the presence of Ag(I). Addition of Ag(I) also caused an inhibitory effect on microbial Fe(II) oxidation by *Acidiplasma* sp. Fv-Ap, resulting in further increase in Fe(II) concentration partly owing to chemical Fe(III) reduction by Ag_2S as described in Eq. 7.5.

Effect of Ag(I) addition on chalcopyrite bioleaching at initial pH of 1.6

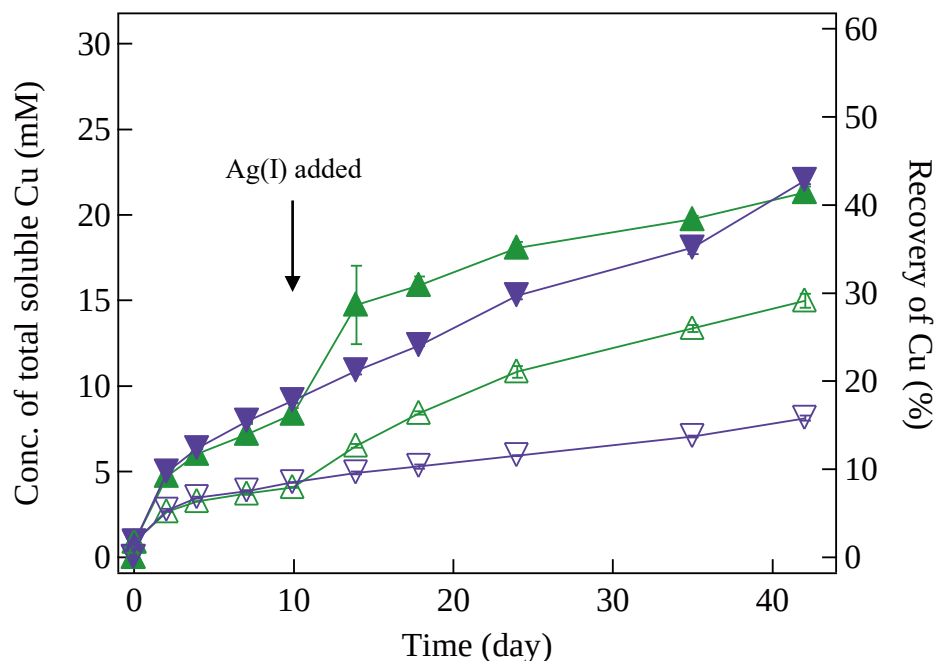


Fig. 7.30 Changes in the concentrations of total soluble Cu leached with (▲, △) and without (▼, ▽) the addition of Ag(I) at day 10, at initial pH of 1.6. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

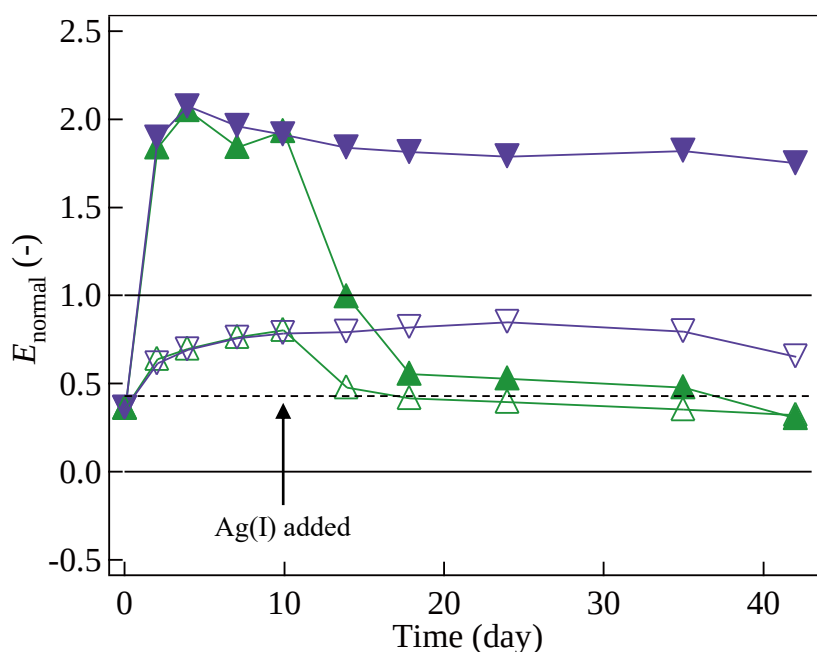


Fig. 7.31 Changes in the E_{normal} values with (▲, △) and without (▼, ▽) the addition of Ag(I) at day 10, at initial pH of 1.6. Open and filled symbols indicate mixed cultures and sterile controls, respectively. Dotted line; $E_{normal}=0.43$.

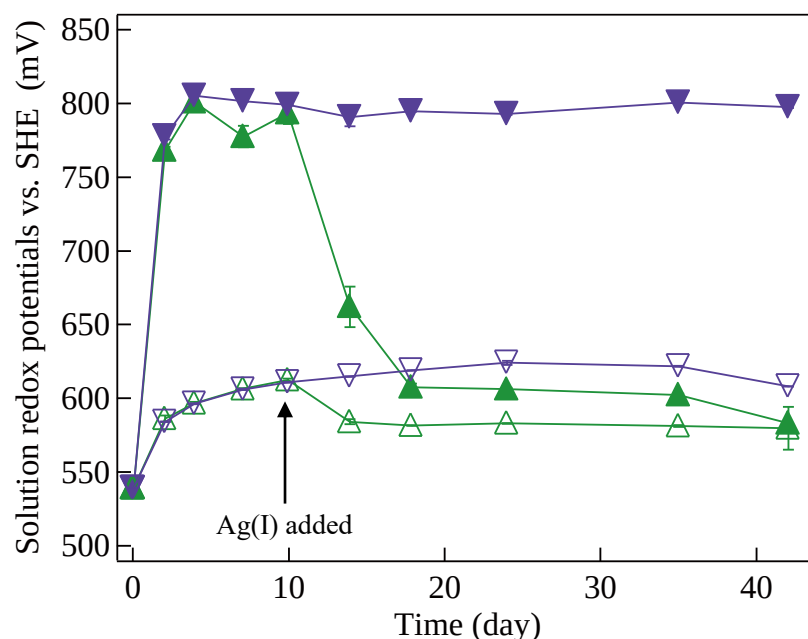


Fig. 7.32 Changes in the solution redox potentials with (▲, △) and without (▼, ▽) the addition of Ag(I) at day 10, at initial pH of 1.6. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

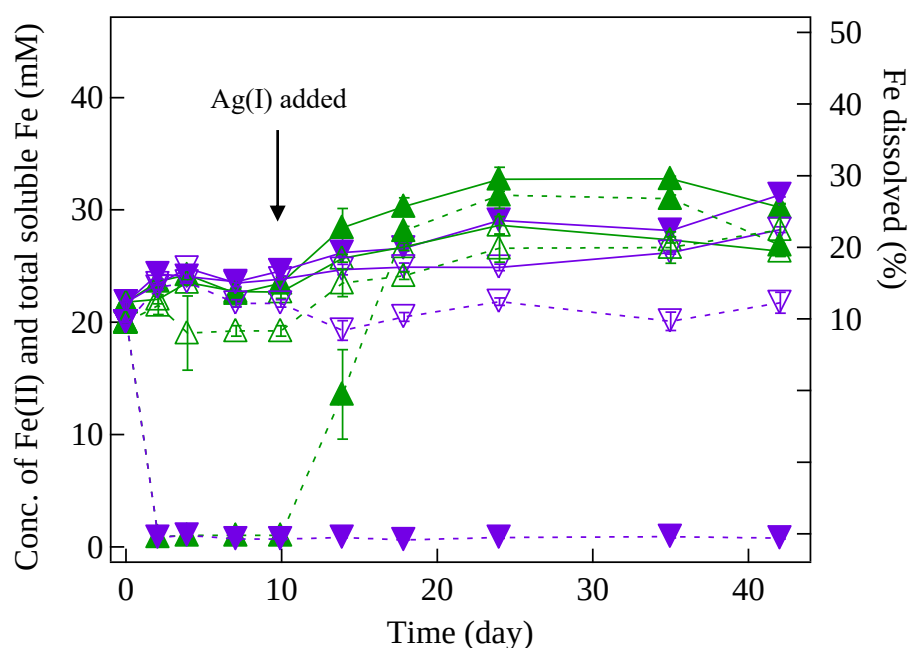


Fig. 7.33 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) leached with (▲, △) and without (▼, ▽) the addition of Ag(I) at day 10, at initial pH of 1.6. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

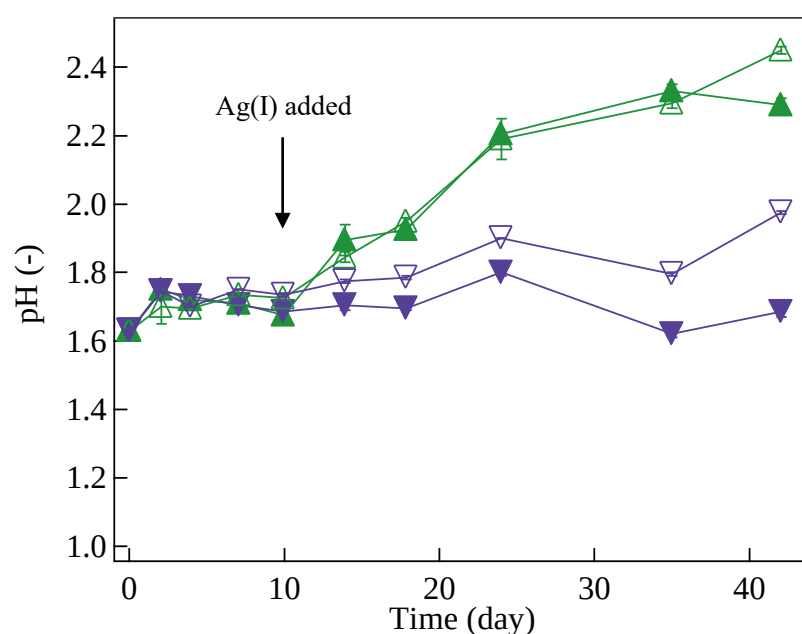


Fig. 7.34 Changes in the pH values with (▲, △) and without (▼, ▽) the addition of Ag(I) at day 10, at initial pH of 1.6. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

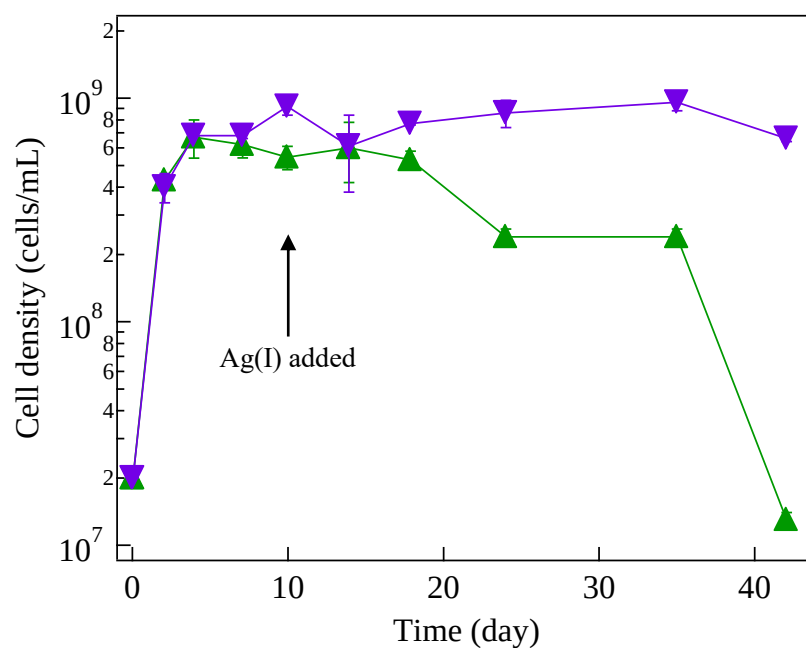


Fig. 7.35 Changes in the cell density with (▲) and without (▼) the addition of Ag(I) at day 10, at initial pH of 1.6.

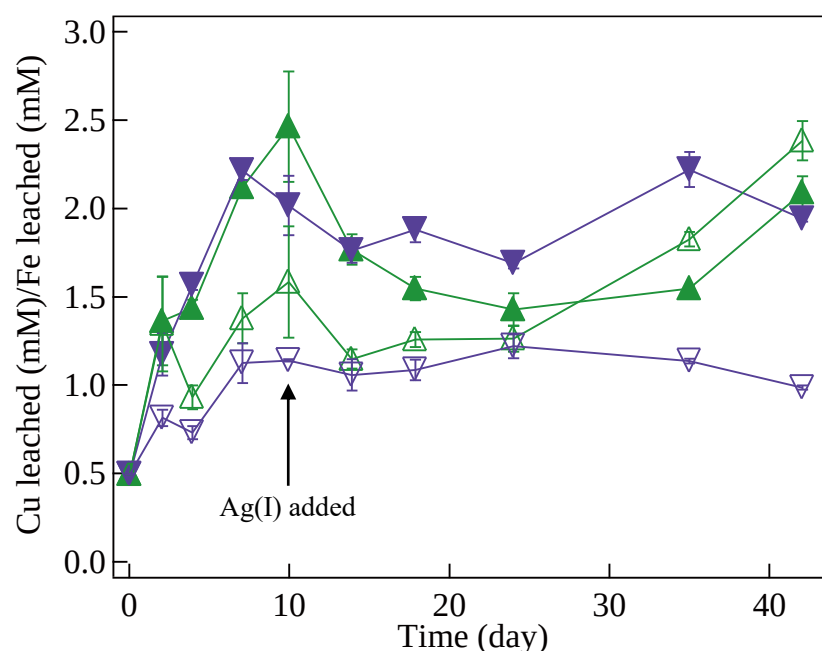


Fig. 7.36 Changes in the molar ratio between Cu and Fe leached with (▲, △) and without (▼, ▽) the addition of Ag(I) at day 10, at initial pH of 1.6. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

(iii) Cu recovery in the absence of Ag(I) at initial pH of 1.6:

In sterile controls, the effect of initial pH was not observed: At both initial pHs, final Cu recovery at day 42 was around 17%. Furthermore, improvement in Cu recovery was also not observed in mixed cultures: At both initial pHs, final Cu recovery at day 42 was 43%, implying that consumption of protons in both mixed cultures (regardless of the initial pHs) were almost same. Since pH values in mixed cultures at the initial pH of 1.3 or 1.6, increased finally to 1.455 or to 1.655, respectively, the total proton consumption in the former can be calculated to be 0.0147 M, but in the latter to be 0.0028 M. This indicates that S^0 -oxidizing activity of *At. caldus* KU was found to be suppressed at lower initial pH. The fact seems to be reasonable since *At. caldus* KU grows well on S^0 at optimal pH of 2.5.

(iv) Cu recovery in the presence of Ag(I) at initial pH of 1.6:

After the addition of Ag(I) at day 10, Cu extraction rate in mixed cultures and sterile controls was similar each other. Furthermore, their changes in the pH values was

also similarly behaved throughout the experiment. This implies that microbial activity of Fe(II) oxidation and S^0 oxidation was inhibited by the presence of Ag(I). In fact, cell density in mixed cultures decreased after the addition of Ag(I).

Regardless of the initial pHs (1.3 or 1.6), Cu dissolution behaviors in mixed cultures was similar each other throughout the experiment, resulting in almost same final Cu recovery (41%) at day 42. In the presence of Ag(I), higher Cu recovery was confirmed when tested at initial pH of 1.3 (52% in Cu recovery) rather than 1.6 (29% in Cu recovery), although effect of initial pHs to final Cu recovery (16-18%) was not observed in the absence of Ag(I). This implies that chalcopyrite dissolution via catalytic reaction of Ag(I) was facilitated at lower pHs. At this point, further experiments are necessary to elucidate this phenomenon.

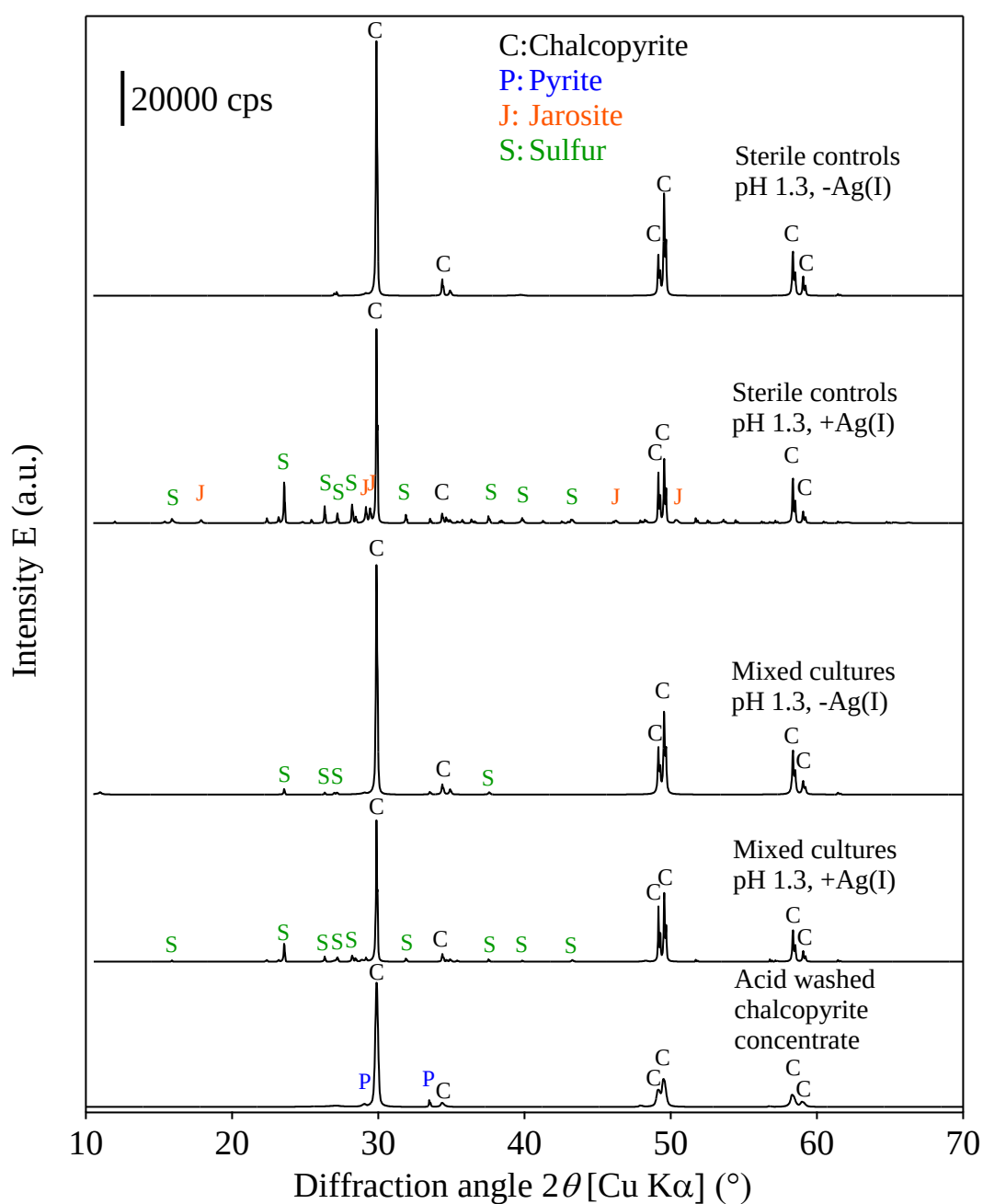


Fig. 7.37 X-ray diffraction patterns of original chalcopyrite concentrate, residual mineral in mixed cultures and sterile controls with and without the addition of Ag(I), respectively, at the initial pH of 1.3 from the bottom to the top. “C”, “P”, “S”, and “J” indicate the peak patterns corresponding to chalcopyrite, pyrite, sulfur, and potassium jarosite, respectively.

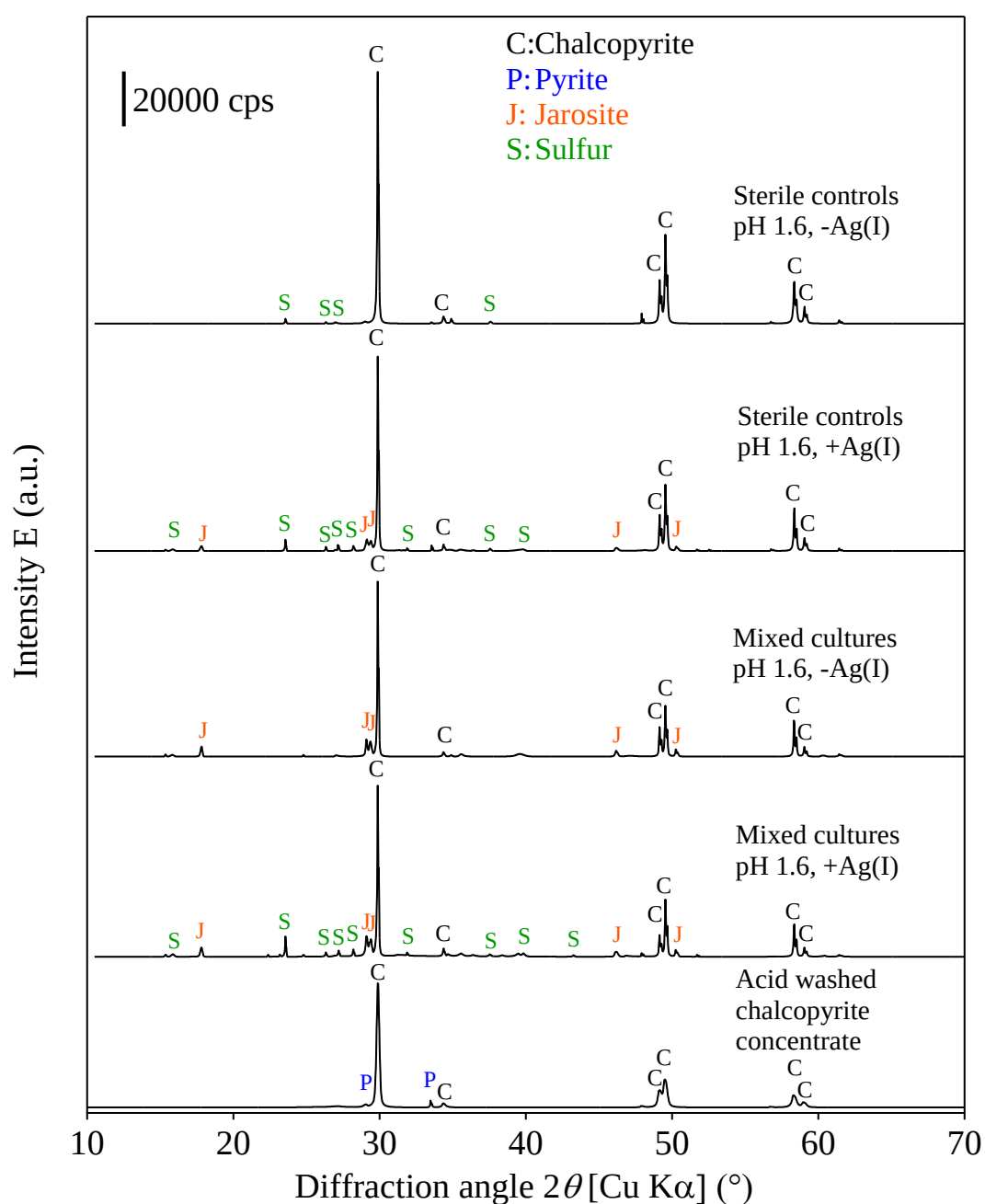


Fig. 7.38 X-ray diffraction patterns of original chalcopyrite concentrate, residual mineral in mixed cultures and sterile controls with and without the addition of Ag(I), respectively, at the initial pH of 1.6 from the bottom to the top. “C”, “P”, “S”, and “J” indicate the peak patterns corresponding to chalcopyrite, pyrite, sulfur, and potassium jarosite, respectively.

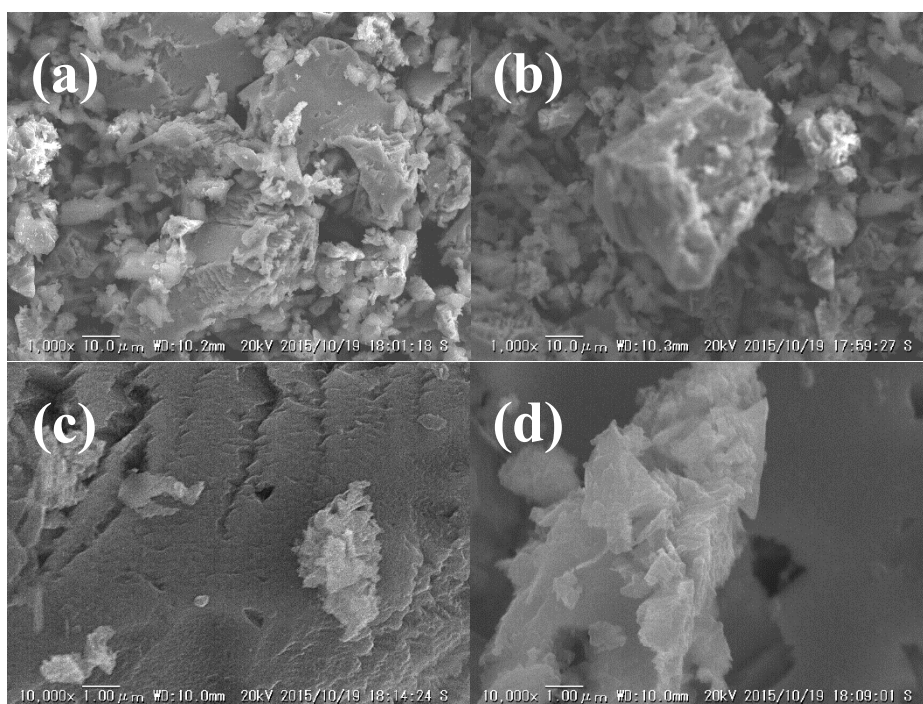


Fig. 7.39 SEM images of bioleached residues in mixed cultures with the addition of Ag(I) at day 10, at initial pH of 1.3. (a) and (b) indicate the images at magnification of 1,000 $\times$. (c) and (d) indicate the images at magnification of 10,000 $\times$.

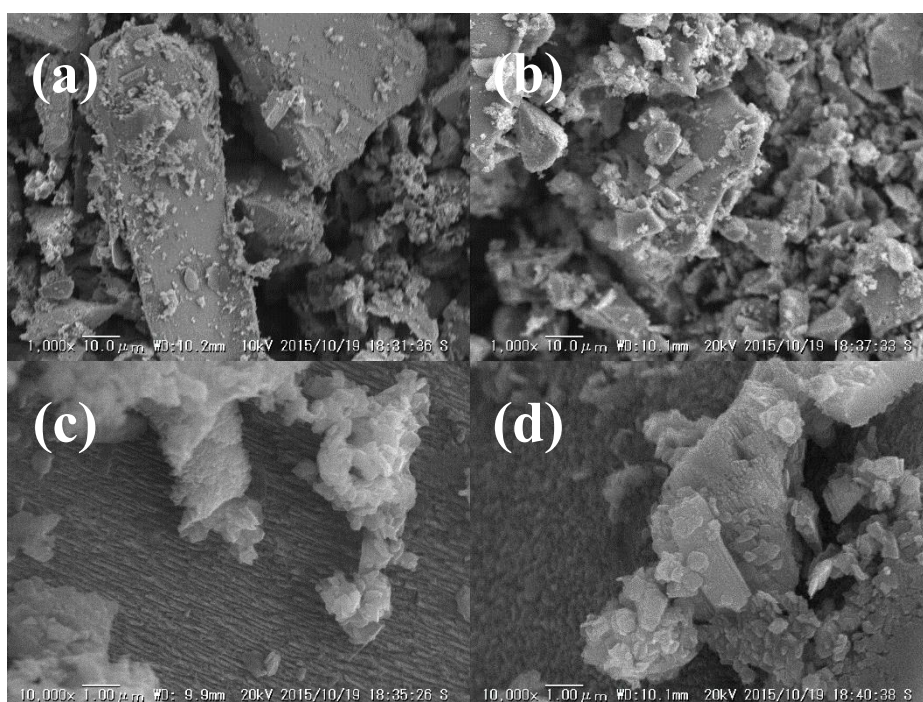


Fig. 7.40 SEM images of bioleached residues in mixed cultures without the addition of Ag(I) at day 10, at initial pH of 1.3. (a) and (b) indicate the images at magnification of 1,000 $\times$. (c) and (d) indicate the images at magnification of 10,000 $\times$.

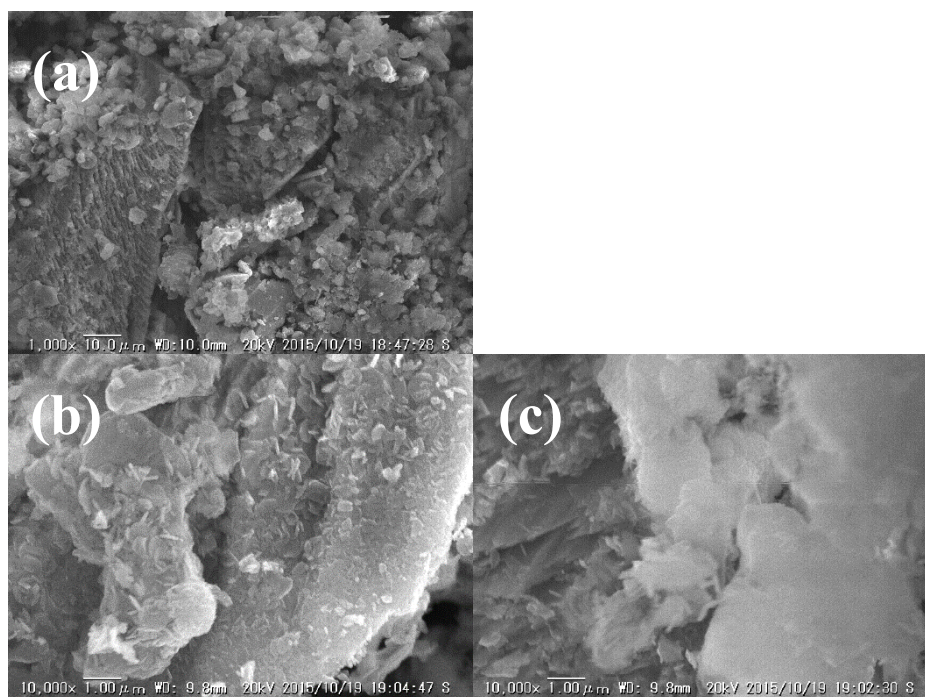


Fig. 7.41 SEM images of bioleached residues in sterile controls with the addition of Ag(I) at day 10, at initial pH of 1.3. (a) indicates the image at magnification of 1,000×. (b) and (c) indicate the images at magnification of 10,000×.

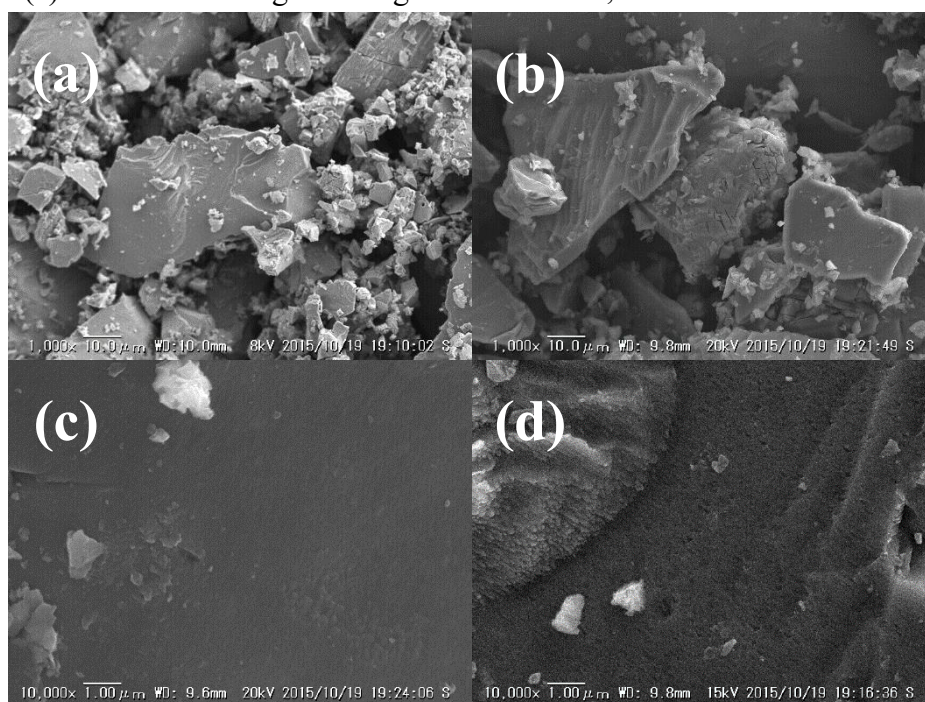


Fig. 7.42 SEM images of bioleached residues in sterile controls without the addition of Ag(I) at day 10, at initial pH of 1.3. (a) and (b) indicate the images at magnification of 1,000×. (c) and (d) indicate the images at magnification of 10,000×.

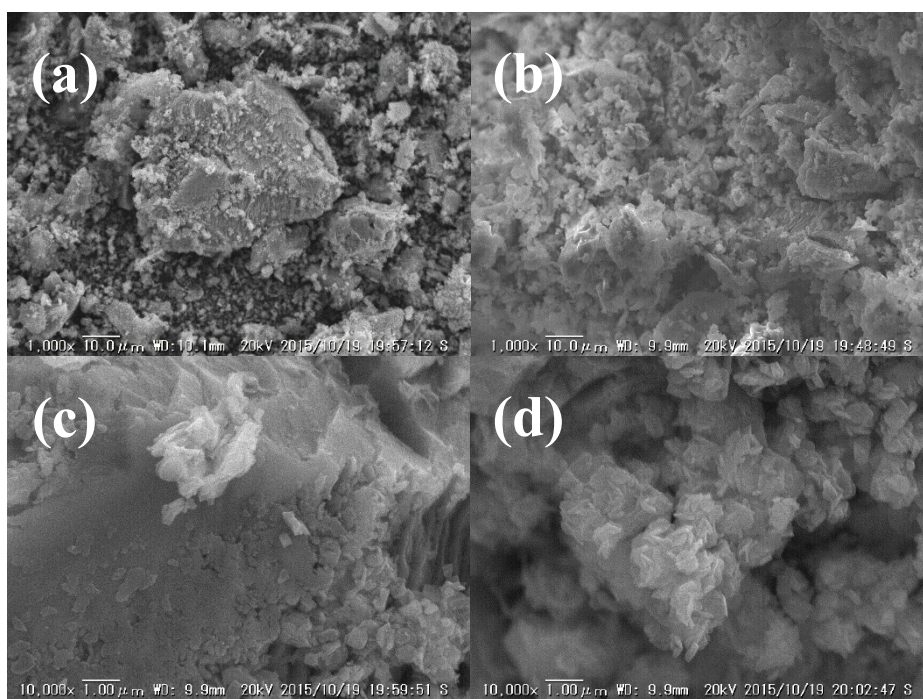


Fig. 7.43 SEM images of bioleached residues in mixed cultures with the addition of Ag(I) at day 10, at initial pH of 1.6. (a) and (b) indicate the images at magnification of 1,000×. (c) and (d) indicate the images at magnification of 10,000×.

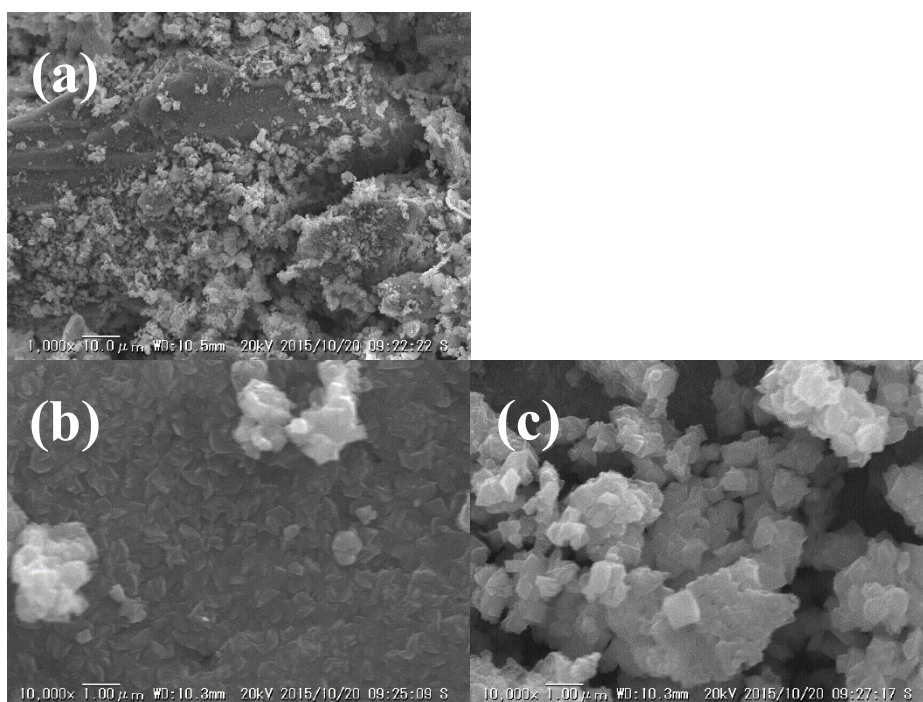


Fig. 7.44 SEM images of bioleached residues in mixed cultures without the addition of Ag(I) at day 10, at initial pH of 1.6. (a) indicates the image at magnification of 1,000×. (b) and (c) indicate the images at magnification of 10,000×.

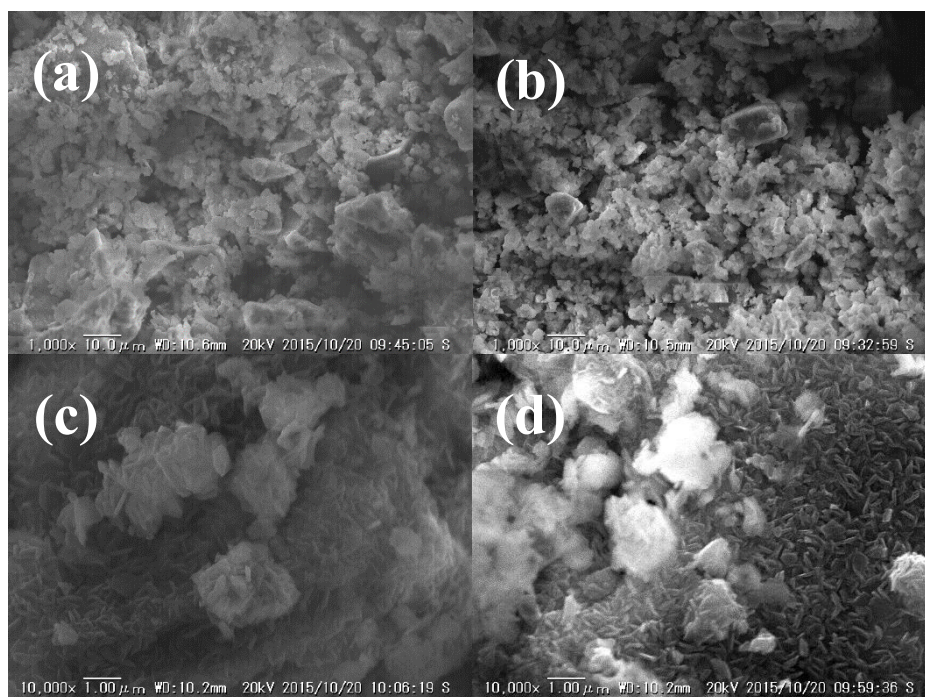


Fig. 7.45 SEM images of bioleached residues in sterile controls with the addition of Ag(I) at day 10, at initial pH of 1.6. (a) and (b) indicate the images at magnification of 1,000×. (c) and (d) indicate the images at magnification of 10,000×.

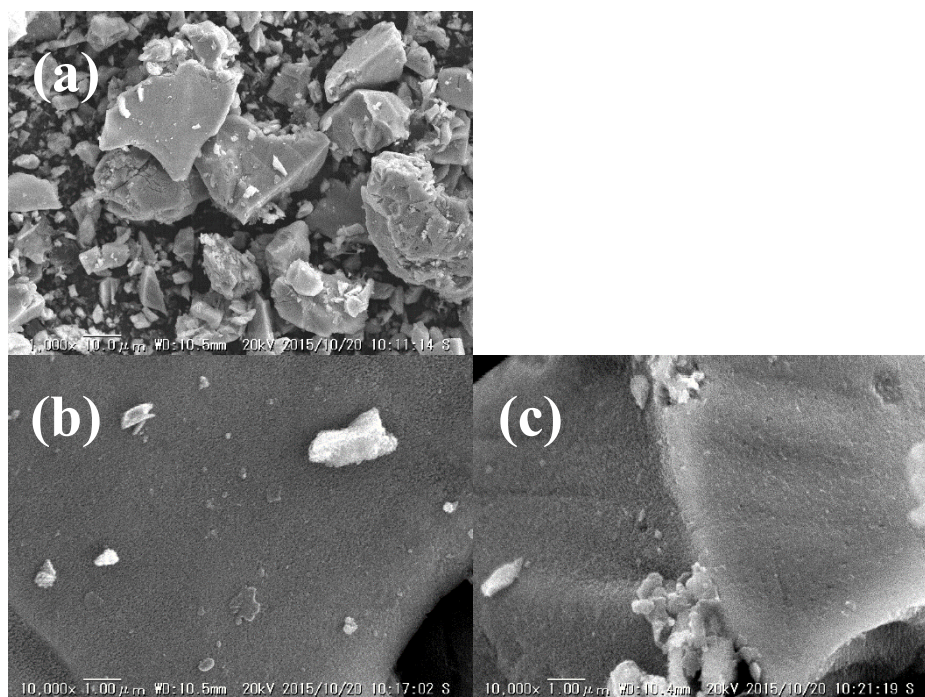


Fig. 7.46 SEM images of bioleached residues in sterile controls without the addition of Ag(I) at day 10, at initial pH of 1.6. (a) indicates the image at magnification of 1,000×. (b) and (c) indicate the images at magnification of 10,000×.

Table 7.1 Summary of the chalcopyrite bioleaching with/without the addition of Ag(I) at different initial pHs.

Initial pHs	Microbial systems	Addition of Ag(I)	[Cu] (mM) at day 42	Cu (%) at day 42	Cu leached (mM)/Fe leached (mM) at day 42
pH 0.6	Strain Fv-Ap	5 mg/L	31	61	1.2
		-	17	32	1.2
	-	5 mg/L	53	100	1.1
		-	9.2	18	1.2
pH 1.3	Strains Fv-Ap and KU	5 mg/L	22	43	1.1
		-	22	43	1.0
	-	5 mg/L	27	52	1.3
		-	8.8	17	0.9
pH 1.6	Strains Fv-Ap and KU	5 mg/L	21	41	2.1
		-	22	43	1.9
	-	5 mg/L	15	29	2.4
		-	8.1	16	1.0

Theoretically, the molar ratio in Cu leached/Fe leached in chalcopyrite bioleaching is 1 after its complete dissolution. “Mixed cultures with and without the addition of Ag(I) at initial pH of 1.6” and “sterile controls with the addition of Ag(I) at initial pH of 1.3 and 1.6”, showed the Cu/Fe ratios of 2.1, 1.9, 1.3, and 2.4, respectively (Table 7.1), indicating formation of ferric precipitates. XRD peak patterns corresponding to potassium jarosite were observed only in the leaching residues in the four systems above. SEM observation showed that in the four systems above, jarosite was found to cover surface of chalcopyrite.

During the chalcopyrite leaching, S^0 is generally formed as described in Eq. 7.5-7.7. Silver catalyzed chalcopyrite leaching reaction induces further accumulation of S^0 . Formation of S^0 in the mixed cultures and sterile controls with the addition of Ag(I), was confirmed by both XRD analysis and SEM observation. One of possible reasons for the fact might be inhibitory effect on microbial S^0 -oxidizing activity of *At. caldus* KU by toxic effect of Ag(I) or Ag_2S . In mixed cultures without the addition of Ag(I), formation of S^0 was not observed regardless of the initial pH values (1.3 or 1.6) based on XRD analysis, indicating that *At. caldus* KU cells were active in the absence of Ag(I) or Ag_2S .

7.3 Section 2 Oxidative bioleaching of chalcopyrite in the presence of silver sulfide using mixed cultures of moderately thermophilic acidophiles

7.3.1 Materials and methods

7.3.1.1 Silver catalyzed bioleaching of chalcopyrite using mixed culture of *Sb. sibiricus* N1, *At. caldus* KU, *Am. ferrooxidans* ICP, and *Fp. acidiphilium* Y

Cells of *Sb. sibiricus* N1, *At. caldus* KU, *Am. ferrooxidans* ICP, and *Fp. acidiphilium* Y were pre-grown, harvested at the late-exponential phase by centrifugation (12,000 g, 10 min at 40°C), washed twice with flesh HBS media (pH 2.0 with H₂SO₄; containing no Cl ions). Two hundred-milliliter of HBS media (pH 1.8 with H₂SO₄; containing no Cl ions) containing trace elements and 5 mM Fe(II), was prepared. The final cell density in the media was set to 4×10⁷ cells/mL. The pulp density of acid washed-pyrite and acid washed-chalcopyrite minerals was set to 0.94 and 1.051% (w/v), respectively. Silver sulfide (Ag₂S) was added to the final densities of 0 and 0.04% (w/v). All 500 mL Erlenmeyer flasks containing the above 200 mL experimental media were shaken at 150 rpm and maintained at 45°C. Samples were regularly taken to monitor cell density, pH values, solution redox potentials, and concentrations of Fe(II), total soluble Fe and Cu. All of the experiments were carried out in duplicates. Residues were analyzed by XRD.

7.3.1.2 Silver catalyzed bioleaching of chalcopyrite using mixed culture of *Acidiplasma* sp. Fv-Ap and *At. caldus* KU

Cells of *Acidiplasma* sp. Fv-Ap and *At. caldus* KU were pre-grown, harvested at the late-exponential phase by centrifugation (12,000 g, 10 min at 40°C), washed twice with flesh HBS media (pH 2.0 with H₂SO₄; containing no Cl ions). Two hundred-milliliter of HBS media (pH 2.0 with H₂SO₄; containing no Cl ions) containing 20 mM Fe(II) and 0.01% (w/v) yeast extract, was prepared. The final cell density in the medium was set to 2×10⁷ cells/mL. The pulp density of acid washed-chalcopyrite minerals was set to 1% (w/v). Silver sulfide (Ag₂S) was added to the final density of 0.04% (w/v). All 500 mL Erlenmeyer flasks containing the above 200 mL experimental medium were shaken at 150 rpm and maintained at 45°C. Samples were regularly taken to monitor cell density, pH values, solution redox potentials, and concentrations of Fe(II),

total soluble Fe and Cu. All of the experiments were carried out in duplicates. Residues were analyzed by XRD.

7.3.2 Results and discussion

7.3.2.1 Silver catalyzed bioleaching of chalcopyrite using mixed culture of *Sb. sibiricus* N1, *At. caldus* KU, *Am. ferrooxidans* ICP, and *Fp. acidiphilium* Y

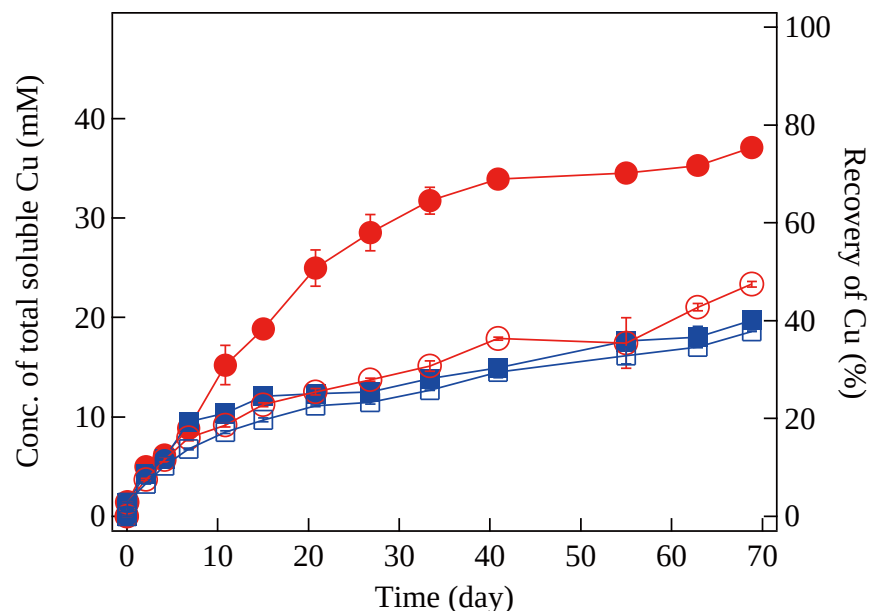


Fig. 7.47 Changes in the concentrations of total soluble Cu leached without (●, ○) and with (■, □) the addition of Ag₂S. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

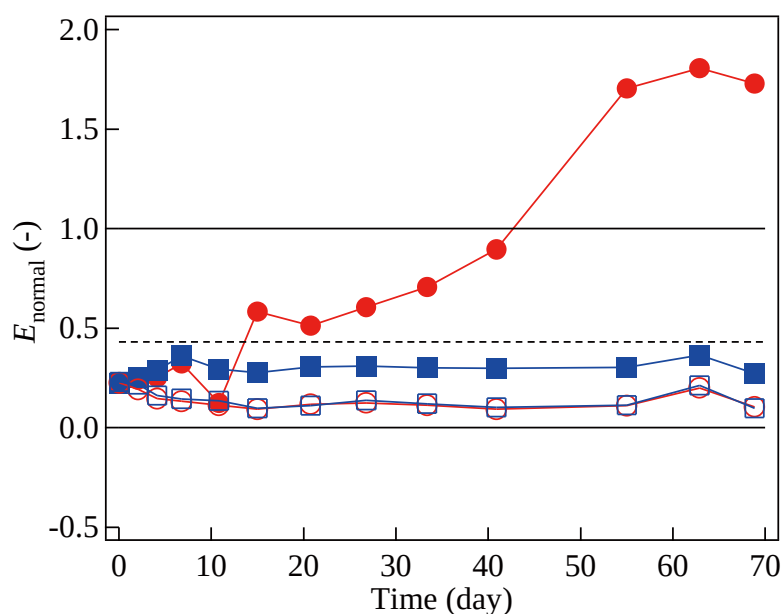


Fig. 7.48 Changes in the E_{normal} values without (●, ○) and with (■, □) the addition of Ag₂S. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

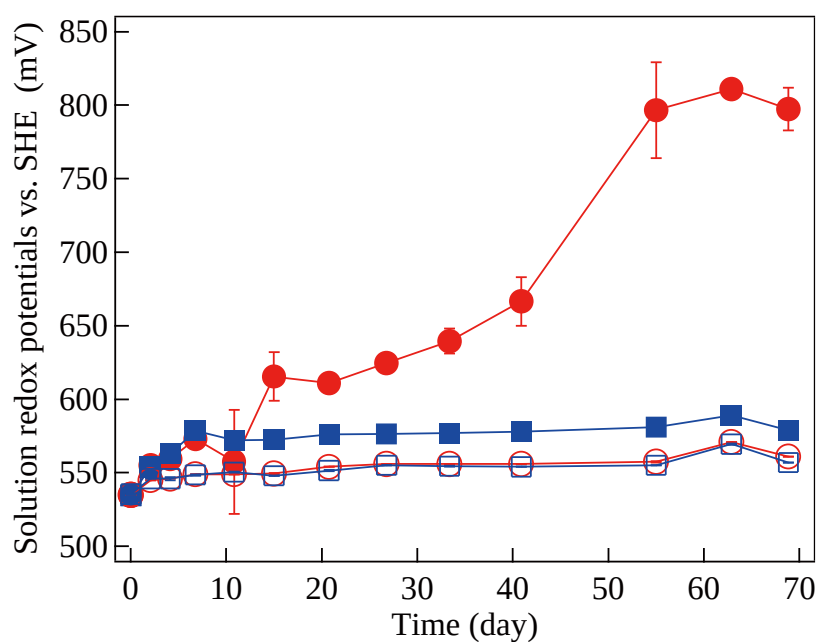


Fig. 7.49 Changes in the solution redox potentials without (●, ○) and with (■, □) the addition of Ag_2S . Open and filled symbols indicate mixed cultures and sterile controls, respectively.

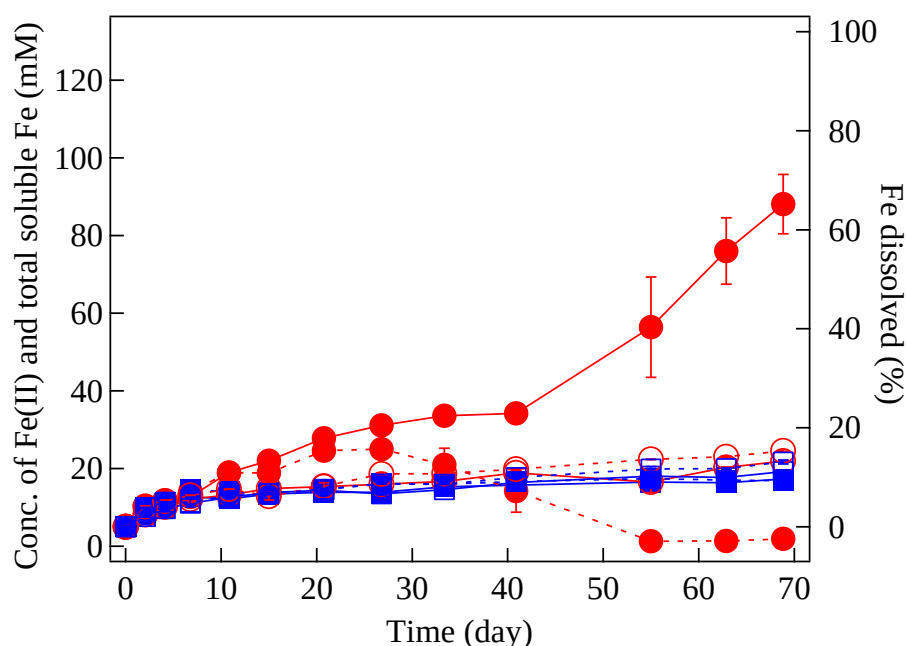


Fig. 7.50 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) leached without (●, ○) and with (■, □) the addition of Ag_2S , respectively. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

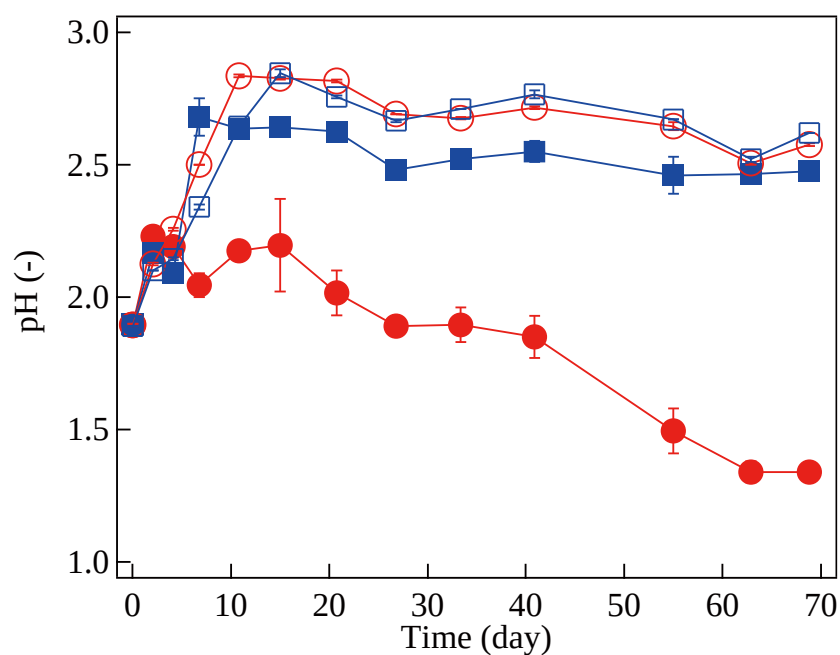


Fig. 7.51 Changes in the pH values without (●, ○) and with (■, □) the addition of Ag₂S. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

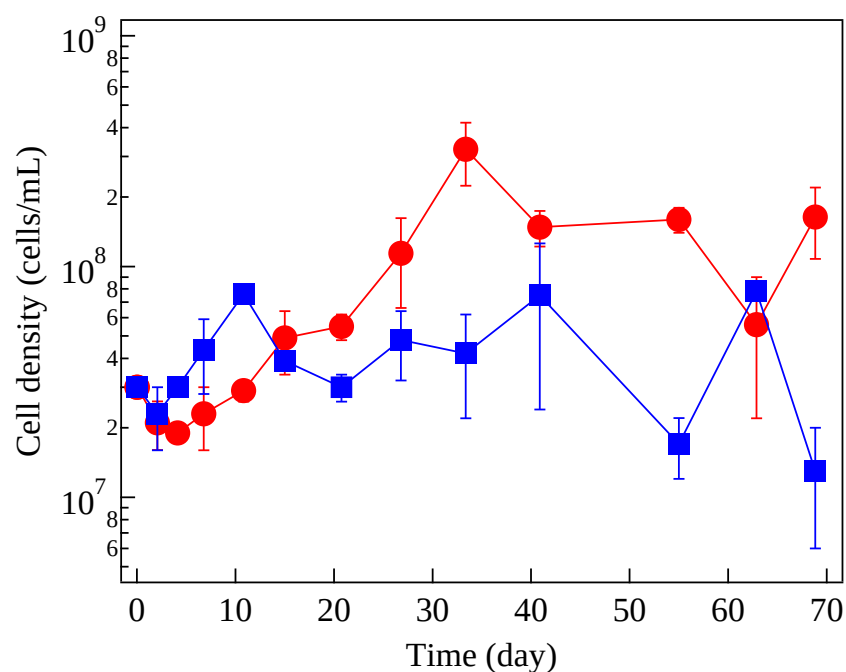


Fig. 7.52 Changes in the cell density without (●) and with (■) the addition of Ag₂S.

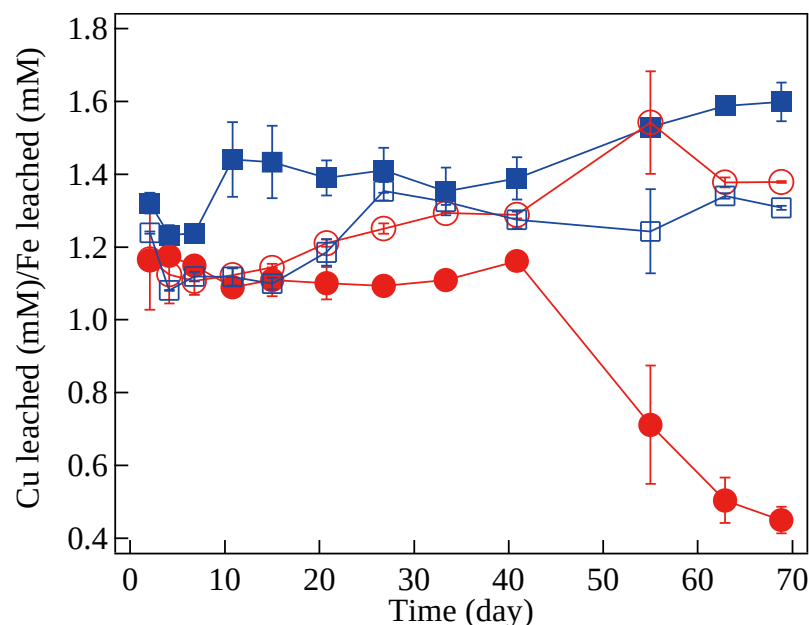


Fig. 7.53 Changes in the molar ration between Cu and Fe leached without (●, ○) and with (■, □) the addition of Ag₂S. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

In sterile controls, addition of Ag₂S to chalcopyrite chemical leaching system did not affect final Cu recovery throughout the experiment: Changes in dissolution of Cu and Fe, solution redox potentials, pH values, and even molar ration between Cu leached/Fe leached, were similarly behaved in sterile controls between with and without the addition of Ag₂S. Although sterile controls marked 38 and 47% as Cu recoveries at day 69 with and without the addition of Ag₂S, respectively, mixed cultures with the addition of Ag₂S showed 41% as Cu recovery within the same period. This might be due to inhibitory effect of microbial Fe(II)- and S⁰-oxidizing activities by the presence of Ag₂S, since increase in solution redox potentials and decrease/maintenance in pH values were significantly suppressed.

Unexpectedly, the most efficient Cu recovery (75%) was confirmed in mixed cultures without the addition of Ag₂S. Note that Cu extraction in that mixed cultures at day 41 was already 69%, and after that it was found to decline. This Cu extraction behavior can be explained by the changes in the E_{normal} values in mixed cultures without the addition of Ag₂S: Cu extraction in mixed cultures without the addition of Ag₂S was found to be accelerated until day 41 compared with that in mixed cultures

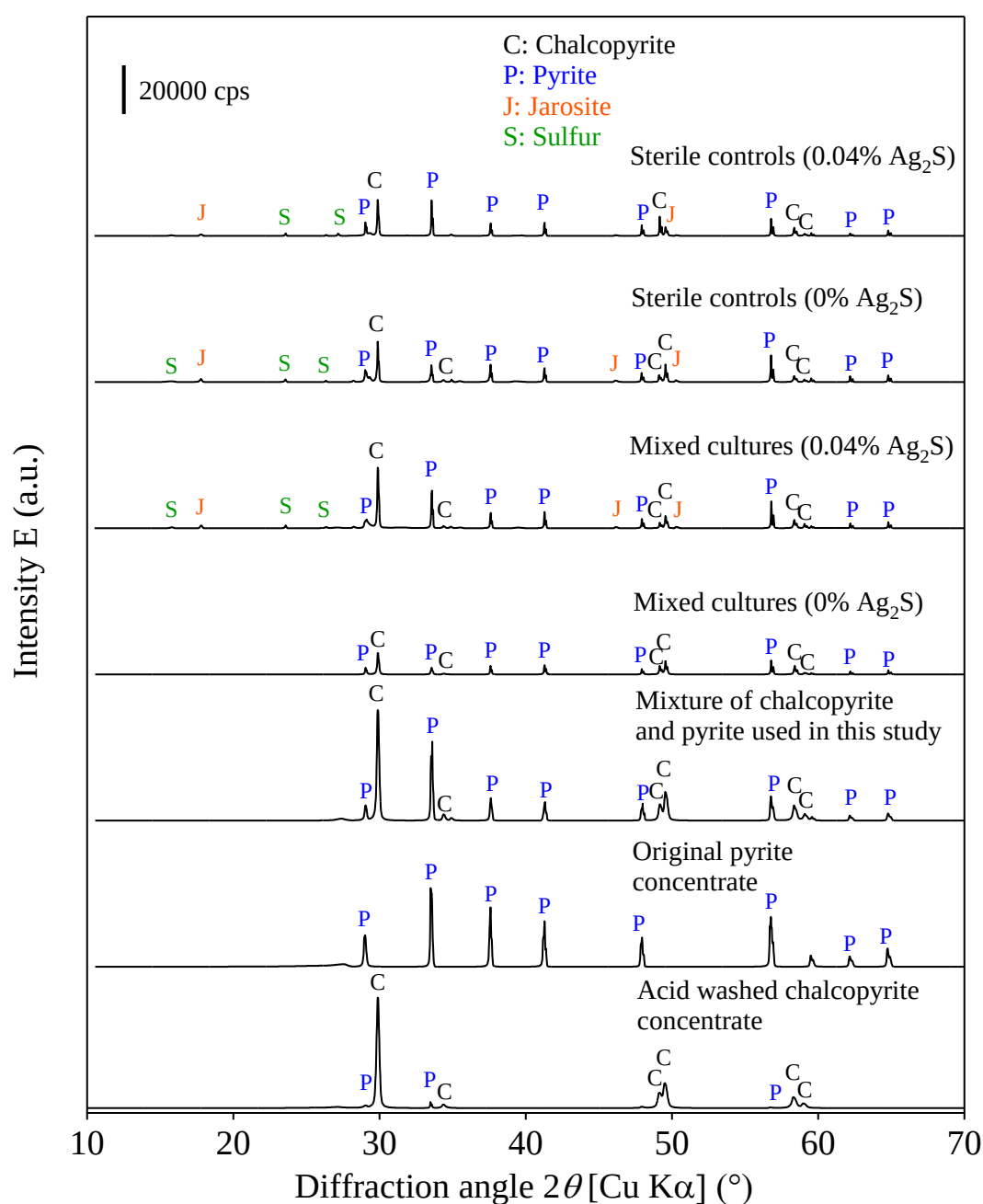


Fig. 7.54 X-ray diffraction patterns of original chalcopyrite concentrate, original pyrite concentrate, mixture of chalcopyrite and pyrite used in this study, residual mineral in mixed cultures and sterile controls without and with the addition of Ag₂S, respectively, from the bottom to the top. “C”, “P”, “S”, and “J” indicate the peak patterns corresponding to chalcopyrite, pyrite, sulfur, and potassium jarosite, respectively.

with the addition of Ag_2S , owing to the the E_{normal} values being maintained within the specific range ($0 < E_{\text{normal}} < 1$). However, Cu extraction rate was declined after the E_{normal} values increased over the range after day 41. Therefore, Cu extraction was found to strongly be affected by the E_{normal} values.

Throughout the bioleaching test, solution redox potentials gradually continuously increased in mixed cultures with the addition of Ag_2S . When solution redox potentials increased to around 800 mV at day 55, Fe dissolution was suddenly boosted, whereas Cu extraction was declined, resulting in rapid decrease in molar ratio of Cu leached/Fe leached from 1.2 to approximately 0.5. This indicates that dissolution of pyrite, but not chalcopyrite, occurred when solution redox potentials increased to around at least 800 mV, as reported by Lowson (1982) and Rodríguez et al (2003); pyrite dissolution activated at solution redox potentials of over 600 mV vs. Ag/AgCl).

XRD analysis showed that peak patterns in sterile controls (with and without the addition of Ag_2S) and mixed cultures with the addition of Ag_2S were similar each other due to their similar leaching behaviors throughout the experiment. In those XRD pattern, accumulation of potassium jarosite and elemental sulfur was observed, caused by increase in pH to 2.5-3.0, and also by inhibitory effect of microbial S^0 -oxidizing activity especially in mixed cultures with the addition of Ag_2S . XRD measurement did not detected the presence of elemental sulfur and potassium jarosite in bioleached residues in mixed cultures without the addition of Ag_2S . Although total soluble Fe concentration reached 88 mM at day 69 (mainly due to pyrite dissolution after day 41), formation of jorasite was not implied. This may be due to the continuous decrease in pH values finally to 1.34 from initial pH of 1.9 within 69 days, contributed oxidation of elemental sulfur to sulfate owing to S^0 -oxidizing activity by *At. caldus* KU.

7.3.2.2 Silver catalyzed bioleaching of chalcopyrite using mixed culture of *Acidiplasma* sp. Fv-Ap and *At. caldus* KU

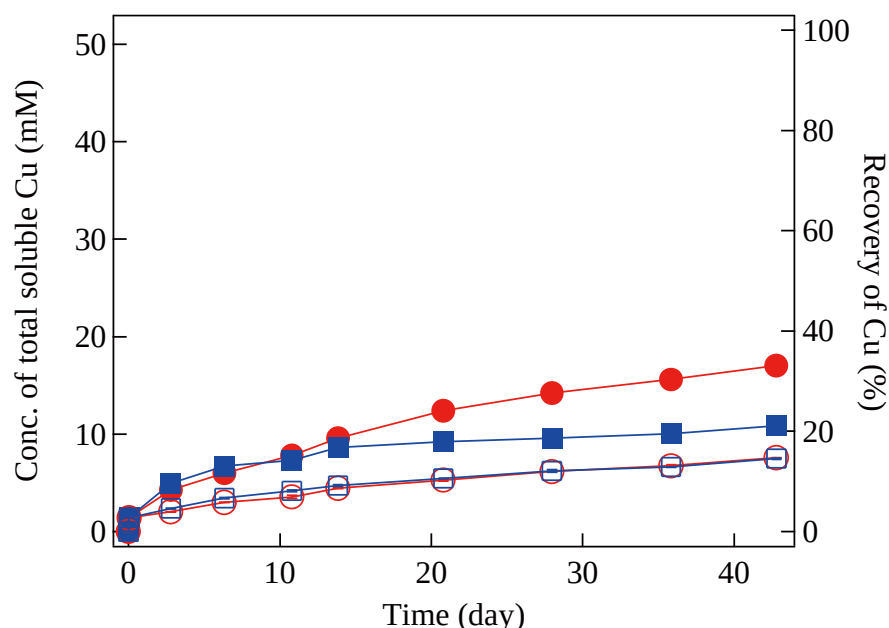


Fig. 7.55 Changes in the concentrations of total soluble Cu leached without (●, ○) and with (■, □) the addition of Ag₂S. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

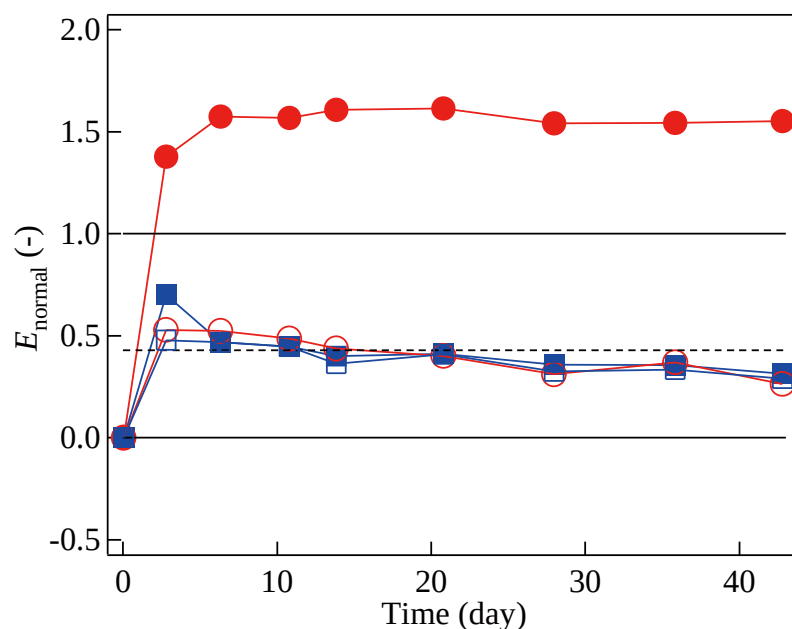


Fig. 7.56 Changes in the E_{normal} values without (●, ○) and with (■, □) the addition of Ag₂S. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

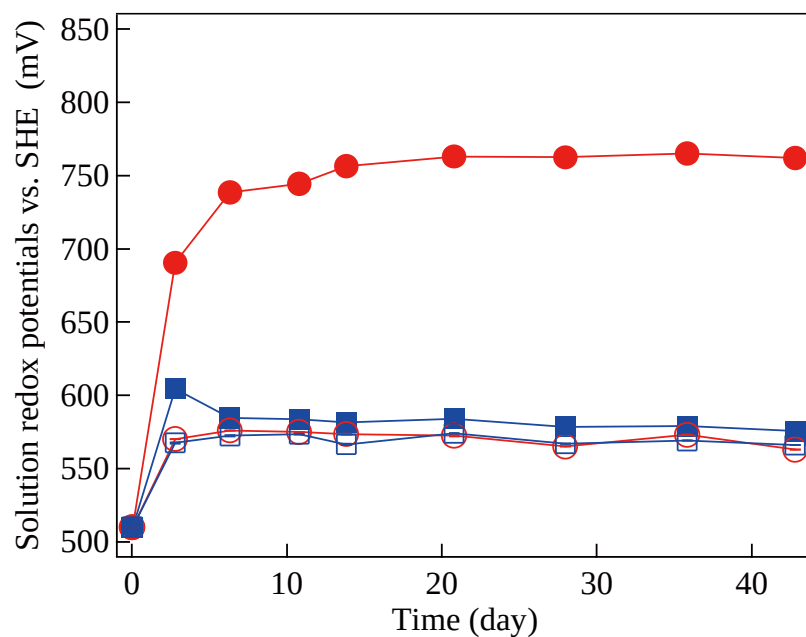


Fig. 7.57 Changes in the solution redox potentials without (●, ○) and with (■, □) the addition of Ag₂S. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

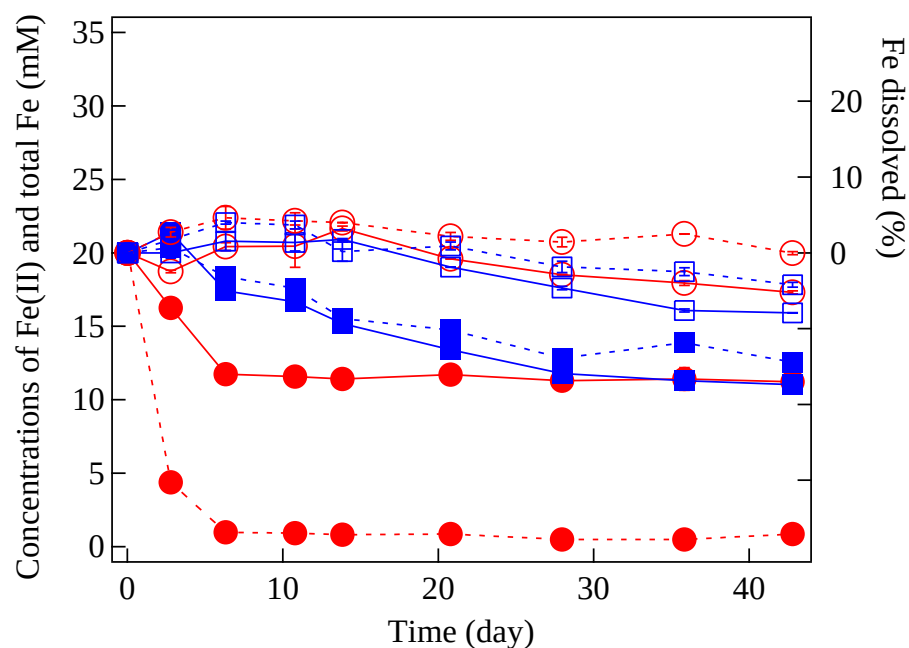


Fig. 7.58 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) leached without (●, ○) and with (■, □) the addition of Ag₂S, respectively. Open and filled symbols indicate mixed cultures and sterile controls, respectively.

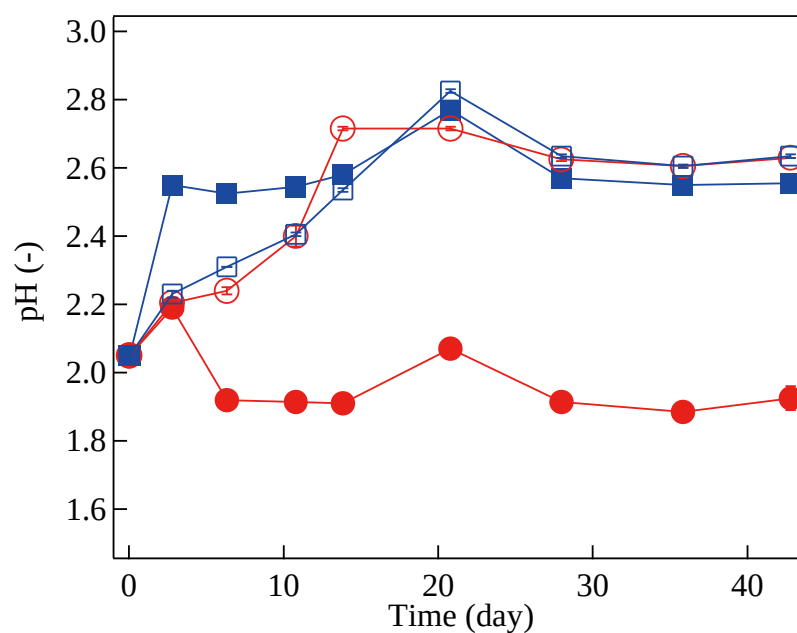


Fig. 7.59 Changes in the pH values without (●, ○) and with (■, □) the addition of Ag_2S . Open and filled symbols indicate mixed cultures and sterile controls, respectively.

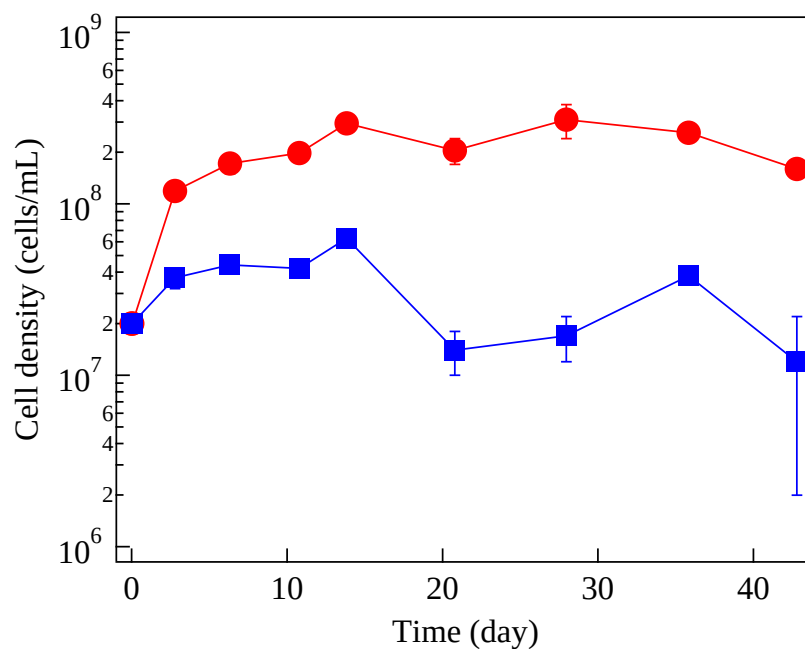


Fig. 7.60 Changes in the solution redox potentials without (●) and with (■) the addition of Ag_2S .

Addition of Ag_2S to chemical leaching system (sterile controls) did not show improvement in Cu recovery as with the sterile controls in the previous experiment. The reason for this may be a lack in soluble Fe(III) ions in sterile controls: Fe(III) are necessary for re-generation of Ag(I) from Ag_2S as shown in Eq. 7.5. Since Fe(II) were dominant species of total soluble Fe throughout the experiment in sterile controls, acceleration of chalcopyrite dissolution via Ag-catalytic reaction seemed not to occur. Inhibitory effect on microbial growth and their Fe(II) - and S^0 -oxidizing activities by the presence of Ag_2S was observed in mixed cultures. In sterile controls (with and without the addition of Ag_2S) and mixed cultures with the addition of Ag_2S , although Cu dissolved to some extent (15-21% at day42), additional accumulation of total soluble Fe over the initial 20 mM Fe(II) added externally, was not observed, resulting in formation of ferric precipitates like jarosite due to their pH values around 2.5. Its formation was also confirmed by XRD patterns. The formation of potassium jarosite might be a reason for the fact that relatively low Cu extraction rate was marked in the sterile controls and mixed cultures discussed above, although the E_{normal} values were maintained at the middle level of the specific range ($0 < E_{\text{normal}} < 1$).

In mixed cultures without the addition of Ag_2S , pH values were maintained at around 2.0 throughout the experiment, indicating that S^0 -oxidizing activity of *At. caldus* KU was active in the bioleaching system. Due to immediate increase in the E_{normal} values (solution redox potentials) to around 1.5 in mixed cultures without the addition of Ag_2S at the early stage of the experiment, Cu extraction from chalcopyrite was suppressed, resulting in only 33% in Cu recovery at day 42.

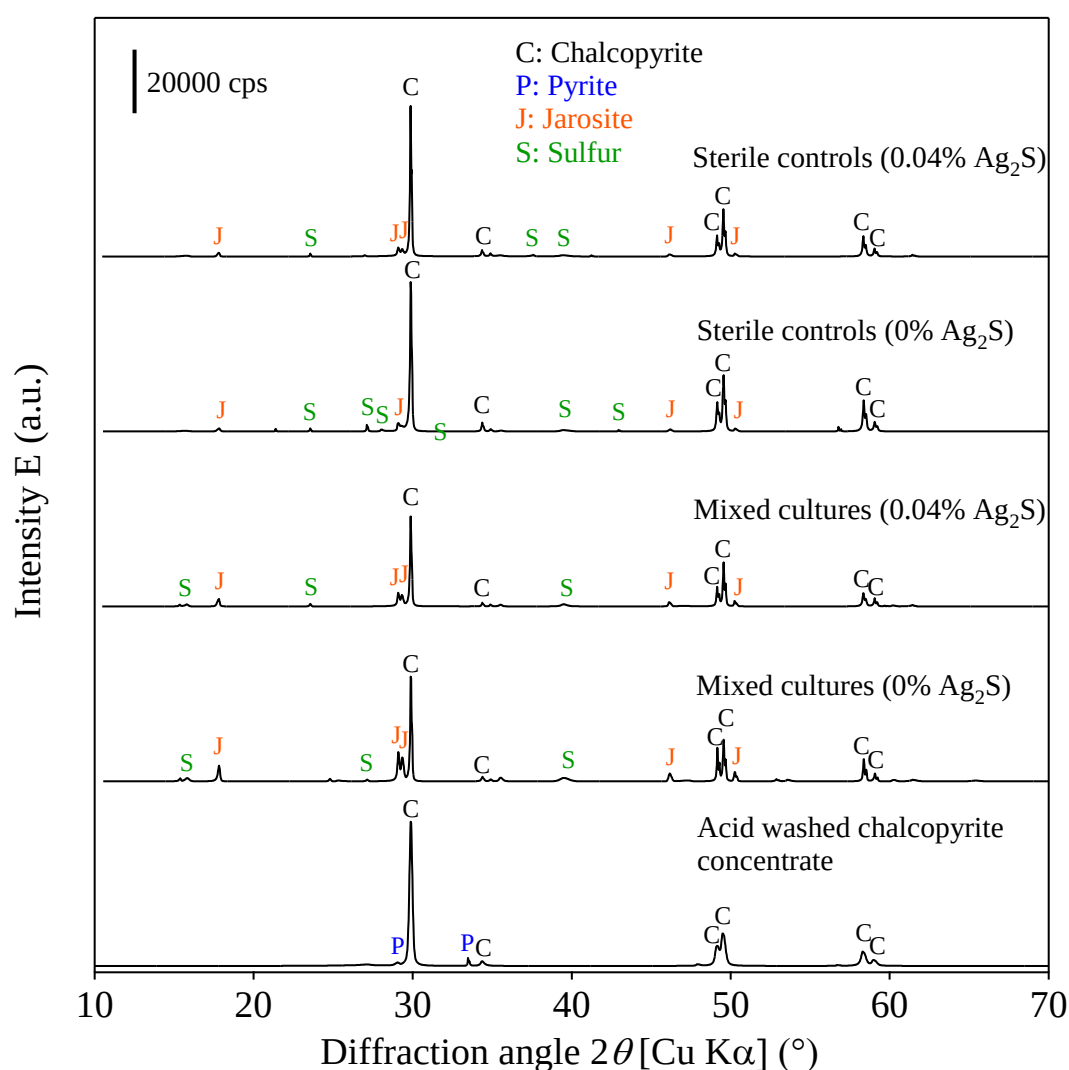


Fig. 7.61 X-ray diffraction patterns of original chalcopyrite concentrate, residual mineral in mixed cultures and sterile controls without and with the addition of Ag_2S , respectively, from the bottom to the top. “C”, “P”, “S”, and “J” indicate the peak patterns corresponding to chalcopyrite, pyrite, sulfur, and potassium jarosite, respectively.

7.4 Conclusions

In section 1, effect of the addition of Ag(I) ions to chalcopyrite bioleaching system on Cu recovery was investigated. Addition of soluble Ag(I) as silver catalyst to chalcopyrite bioleaching system at day 10 enhanced Cu recovery only in sterile controls, while final Cu recovery in mixed cultures reached 43% regardless of the addition of Ag(I). However, Ag(I) inhibited microbial Fe(II)- and S^0 -oxidizing activities of *Acidiplasma* sp. Fv-Ap and *At. caldus* KU, respectively. In the presence of Ag(I), sterile controls showed higher Cu recovery than that in mixed cultures, which might have been caused by accumulation of S^0 on the chalcopyrite surface at the high solution redox potential level in mixed cultures before the addition of Ag(I). Passivation effect of S^0 on the mineral surface could be prevented by adapting *At. caldus* KU to Ag(I).

In this chapter, changes in the E_{normal} values were discussed assuming that chalcopyrite dissolution is based on formation of chalcocite as an intermediate. Since acceleration of Cu extraction was observed when the E_{normal} values were within the range ($0 < E_{\text{normal}} < 1$), especially in the middle level of the range, solution redox potentials may strongly affect chalcopyrite dissolution efficiency. Considering both effect of the E_{normal} values and Ag addition on chalcopyrite dissolution efficiency, a balance between accumulation of Fe(II) through Ag-catalyzed chalcopyrite dissolution (Eq. 7.5 and 7.6) and microbial Fe(II)-oxidizing activity, may be one of the crucial keys, in order to successfully achieve improvement in Cu recovery via the addition of Ag(I) to the chalcopyrite bioleaching system within the specific the E_{normal} range ($0 < E_{\text{normal}} < 1$).

In section 2, effect of the addition of Ag_2S to chalcopyrite bioleaching system on Cu recovery was studied. Unexpectedly, the addition of Ag_2S to bioleaching system resulted in inhibitory effect on microbial activities of Fe(II) and S^0 oxidation. Since 0.04% (w/v) Ag_2S corresponds to 348 mg/L as Ag, the amount of Ag added in the experiments in section 2 (Ag_2S was added) was much larger than that in section 1 (Ag(I) ion was added), its toxic effect on microbial activity in the latter was more critical.

In order to succeed in Ag-catalyzed bioleaching of chalcopyrite, continuous supply of Fe(III) is necessary for re-generation of Ag_2S from Ag(I), as shown in Eq. 7.5 and 7.6. However, Fe(II)-oxidizing acidophiles used in the experiment in chapter 7

was not tolerant to Ag(I) or Ag₂S, critically causing to inefficient Cu recovery in mixed cultures with the addition of Ag catalyst.

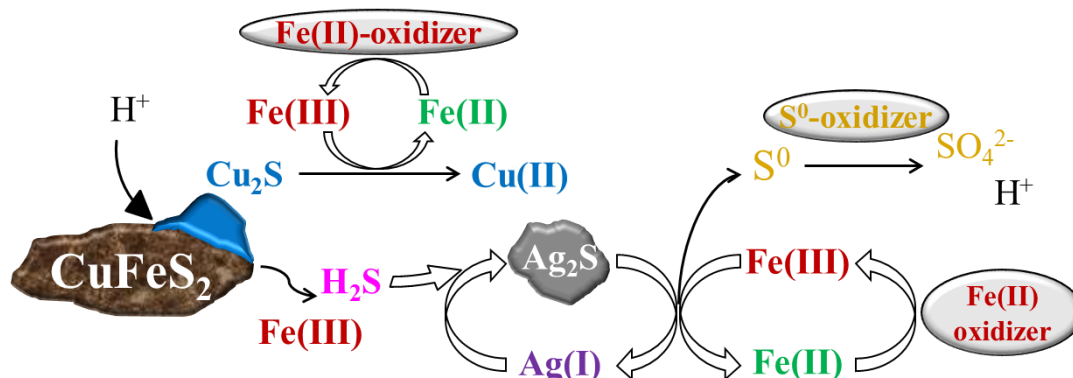


Fig. 7.62 Scheme of silver-catalyzed chalcopyrite bioleaching system and inhibitory effect on microbial activities by the presence of Ag(I).

In addition to the effect of Ag addition on chalcopyrite bioleaching efficiency, changes in the E_{normal} values critically affected Cu recovery, especially selective dissolution of chalcopyrite without pyrite dissolution at the specific range; $0 < E_{\text{normal}} < 1$. Enhancement in Cu extraction rate at the range $0 < E_{\text{normal}} < 1$, especially in the middle of the range, even in the presence of Ag(I) and Ag₂S, was confirmed in this chapter, indicating that two different mechanisms of chalcopyrite dissolution are involved in the presence of Ag catalyst; (i) one is chalcopyrite dissolution via Ag catalytic reaction and (ii) the other is its dissolution via formation and oxidation of intermediate, chalcocite (based on the E_{normal} values). Overall results indicate that a balance between Fe(II) accumulation via the former mechanism and microbial Fe(II)-oxidizing activity that can maintain the E_{normal} levels at the specific range ($0 < E_{\text{normal}} < 1$), is important in order to maximize Cu recovery in Ag catalyzed-bioleaching system.

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Chapter 8

Microbiological solution redox potential control in the chalcopyrite bioleaching system for effective Cu recovery

Abstract

Further development of copper (Cu) extraction techniques from low-grade refractory sulfides (e.g., chalcopyrite) has actively been studied in order to maximize its Cu extraction in hydrometallurgical system. Involvement of chalcocite as an intermediate in the chalcopyrite chemical leaching has recently been proposed, and higher Cu extraction rate has been confirmed at relatively low solution redox potentials, rather than at relatively high. This study investigated the potential utility of the microbiological solution redox potential control in the chalcopyrite bioleaching system for maximization of Cu recovery. Utilization of different microbial Fe(II)-oxidizers with different degree of Fe(II)-oxidizing capability to the chalcopyrite bioleaching test resulted in different levels of E_{normal} values. Recovery of Cu in mixed cultures A (*Acidithiobacillus (At.) caldus* KU mixed with *Sulfobacillus acidophilus* YTF1) and B (*At. caldus* KU mixed with *Acidiplasma* sp. Fv-Ap) reached 75 and 49%, respectively, at day 70. The difference in the final Cu recovery in the two mixed cultures was attributed to the solution redox potential levels during bioleaching: The E_{normal} values in the former were within the range of $0 < E_{\text{normal}} < 1$, whereas those in the latter were $E_{\text{normal}} > 1$. In this study, Cu extraction rate was found to be maximized at $0 < E_{\text{normal}} < 1$, especially in the middle of the range, rather than at $E_{\text{normal}} > 1$.

8.1 Introduction

Enhancement of Cu recovery in chalcopyrite bioleaching system at relatively low redox potential level, rather than at higher redox level, was reported. Hiroyoshi et al (2000, 2001) reported that chalcopyrite dissolution proceeds via formation of chalcocite (Cu_2S) as an intermediate, and is strongly affected by solution redox potentials: The formation (Eq. 8.1) and oxidation (Eq. 8.2) of chalcocite was found to facilitate dissolution of chalcopyrite as follows:



Okamoto et al (2004) defined “ E_{normal} ” to evaluate optimal redox potential levels for the chalcopyrite leaching system, and proposed that chalcopyrite dissolution can be maximized at the E_{normal} value of 0.43.

$$E_{\text{normal}} = (E - E_{\text{ox}}) / (E_{\text{c}} - E_{\text{ox}}) \quad (\text{Eq. 8.3})$$

$$E_{\text{c}} (\text{V}) = 0.72 + 0.059 \log \frac{(a_{\text{Cu}^{2+}})^{0.75}}{(a_{\text{Fe}^{2+}})^{0.25}} \quad (\text{Eq. 8.4})$$

$$E_{\text{ox}} (\text{V}) = 0.60 + 0.059 \log (a_{\text{Cu}^{2+}})^{0.5} \quad (\text{Eq. 8.5})$$

, where R , T , F , and a_i are gas constant (J/Kmol), temperature (K), faraday constant (C/mol), and the activities of species i . E_{c}^0 and E_{ox}^0 indicate the standard redox potentials (V) of Eq. 8.1 and 8.2, respectively.

Owing to several advantages with bioleaching techniques (e.g., applicable to refractory lower Cu grade ores, with relatively low cost) for the practical Cu extraction process, chalcopyrite bioleaching has been actively studied to improve its efficiency. However, the effect of the redox potential level on the chalcopyrite bioleaching system has yet been studied in detail. This study thus aimed to investigate how solution redox potentials affect Cu recovery efficiency in bioleaching systems.

The dissolution kinetics of chalcopyrite in bio/chemical leaching systems have been discussed through shrinking core model, as follows (Aydogan et al 2006):



When the dissolution reaction is controlled by diffusion through fluid film, the dissolution model can be described as follows (Gbor et al 2004):

$$X = \frac{3bk_g C_{Ab}}{\rho R^2} t = k_m t \quad (\text{Eq. 8.7})$$

When the dissolution reaction is controlled by diffusion through solid product film, the dissolution model can be described as follows (Gbor et al 2004):

$$1 - 3(1 - X)^{2/3} + 2(1 - X) = \frac{6bDC_{Ab}}{\rho R^2} t = k_d t \quad (\text{Eq. 8.8})$$

When the reaction is controlled by the surface chemical reactions, the dissolution model can be expressed as follows (Gbor et al 2004):

$$1 - (1 - X)^{1/3} = \frac{bk_c C_{Ab}^n}{\rho R} t = k_r t \quad (\text{Eq. 8.9})$$

, where X is the fraction of B reacted, b is the moles of solid B consumed per mole of fluid A reacted, D is the diffusion coefficient in the product-layer, C_{Ab} is the concentration of soluble A in the bulk solution, ρ is the density of solid, R is the radius of solid particle B, t is the reaction time, k_g is mass transfer coefficient of A in the fluid film, k_c is the chemical reaction constant, n is the order of reaction with respect to A, k_m , k_d and k_r are the rate constants of each model.

Under conditions where chalcopyrite is oxidized in sulfate solution, the dissolution kinetics tend to depend on oxidant types; Fe(III), or non- Fe(III) (e.g., Cr(VI), H₂O₂, etc.). In bioleaching system, Fe(III) is important oxidant that is generally dominant species in total soluble Fe due to microbial Fe-oxidizing activity. Therefore,

the chalcopyrite dissolutions in bioleaching system have been recognized to be controlled by diffusion through solid product film since jarosite is often formed and accumulated on the mineral surface due to relatively higher Fe(III) concentration (Jordan et al 2006, Pradhan et al 2010, Abhilash et al 2013, Panda et al 2013, Abhilash and Pandey 2015). Al-Harashsheh et al (2005) reported that chalcopyrite dissolution using 0.25 M_{ini} Fe(III) as the oxidant was controlled by surface chemical reaction, which might have been caused by hyper acidic solution (0.5 M H₂SO₄). Kaplun et al (2011) reported that chalcopyrite dissolution without the addition of Fe(III) was controlled by surface chemical reaction even with redox potential control at 750 mV (vs. SHE). On the other hand, When the above non-Fe(III) oxidants were utilized for chalcopyrite oxidation, the chalcopyrite dissolutions have been reported to be controlled by surface chemical reaction (Murr and Hiskey 1981, Antonijevic et al 1994, 2004, Adebayo et al 2003, Mahajan et al 2007). This might have been due to less amount of Fe(III) generated during the chemical leaching, compared with that in bioleaching system. Aydogan et al (2006) contradictory proposed that chalcopyrite dissolution using potassium dichromate as oxidant was controlled by diffusion through solid product film. One of the possible reasons why it did not follow surface chemical reaction would be formation of PbSO₄ due to impurity of PbS in chalcopyrite concentrate used in that study.

The information about redox dependence of chalcopyrite dissolution in bioleaching system that redox potential is not controlled electrochemically is yet highly limited, and evaluation of the chalcopyrite leaching behaviors using E_{normal} values was only reported by electrochemical and chemical leaching experiments, but not by bioleaching test. Therefore, chalcopyrite bioleaching behaviors was evaluated through E_{normal} values in this study. Furthermore, solution redox potential level was microbiologically controlled using different degree of Fe(II)-oxidizing capabilities deriving from different Fe(II)-oxidizers.

In this study, mixotrophic S⁰-oxidizing acidophile, *Acidithiobacillus* (*At.*) *caldus* strain KU was used as a S⁰-oxidizer (the strain is able to grow autotrophically on elemental sulfur; Hallberg and Lindström 1994). *Sulfobacillus* (*Sb.*) *acidophilus* strain YTF1, *Sb. sibiricus* strain N1, *Acidimicrobium* (*Am.*) *ferrooxidans* strain ICP, and *Acidiplasma* sp. strain Fv-Ap, were used as moderately thermophilic, mixotrophic

Fe(II)-oxidizing acidophiles (Clark and Norris 1996, Bridge and Johnson 1998, Melamud et al 2003, Golyshina et al 2009). Bridge and Johnson (1998) reported that *Sulfobacillus* spp. are implied to be facultative anaerobes rather than strict aerobes, due to their utilization of Fe(III) as an electron acceptor with either organic and inorganic electron donor in anoxic conditions. Strain YTF1 was implied to show relative Fe-oxidizing/reducing capabilities among the strains in the genus *Sulfobacillus*: Soluble Fe(II)/Fe(III) ratio was maintained at around 1 during Fe oxido-reduction test over 300 hours (Bridge and Johnson 1998). Strain YTF1 was reported to grow on elemental sulfur and Fe(II) autotrophically, as well as heterotrophically on yeast extract (Norris et al 1996). Strain N1 was reported to grow autotrophically on Fe(II), and its Fe(II) oxidation was observed to be more active in the presence of yeast extract (Melamud et al 2003). The strain was also found to grow on elemental sulfur heterotrophically with yeast extract, but not autotrophically. Clark and Norris (1996) reported that strain ICP is able to grow autotrophically on Fe(II) and heterotrophically on yeast extract. The strain was also reported to show complete oxidation of 50 mM Fe(II) rapidly, in the presence of yeast extract (Norris et al 1996). Golyshina et al (2009) reported that the genus *Acidiplasma* is facultative anaerobic, and it oxidizes Fe(II) in the presence of trace amount of yeast extract. Strain Fv-Ap was found to oxidize 40 mM Fe(II) rapidly in our laboratory. Overall, different capabilities of microbial Fe(II) oxidation by the four acidophilic strains is the key for redox potential control in chalcopyrite bioleaching system, and its order is as follows; the weakest for strain YTF1, relatively weaker for strain N1, relatively stronger for strain Fv-Ap, and the strongest for strain ICP.

This study thus aimed (i) to evaluate the potential utility of the microbiological solution redox potential control for maximization of Cu recovery from chalcopyrite concentrate and (ii) to investigate its detailed mechanism from the viewpoint of electrochemistry and kinetic study.

8.2 Materials and methods

8.2.1 Effect of the microbiological control of solution redox potentials on chalcopyrite bioleaching efficiency using mixed cultures of *At. caldus* KU and either *Sb. sibiricus* N1, *Sulfobacillus* sp. YTF1, *Acidiplasma* sp. Fv-Ap, or *Am. ferrooxidans* ICP

Cells of the five strains, *At. caldus* KU, *Sb. sibiricus* N1, *Sulfobacillus* sp. YTF1, *Acidiplasma* sp. Fv-Ap, and *Am. ferrooxidans* ICP, were pre-grown separately as described above, harvested in the late exponential phase by centrifugation ($12,000 \times g$, 10 min, 40°C), washed twice with fresh HBS medium (pH 1.5 with H_2SO_4). All the bioleaching tests in this work were performed in 500 mL Erlenmeyer flasks containing 200 mL HBS medium (pH 2.0 with H_2SO_4) supplemented with 0.01% (w/v) elemental sulfur, 5 mM Fe(II), trace elements and 1% (w/v) acid washed-chalcopyrite concentrate. Five mixed cultures, A (KU and YTF1), B (KU and N1), C (KU and Fv-Ap), D (KU and Fv-Ap in the presence of 0.01% (w/v) y.e.), and E (KU and ICP in the presence of 0.01% (w/v) y.e.), were prepared. The initial cell densities were set to 2×10^7 cells/mL in total (1×10^7 cells/mL of each strain). All the media were maintained at 45°C on an orbital rotary shaker at 150 rpm. Samples were regularly taken to monitor cell density, pH values, solution redox potential values, and concentrations of Fe(II), total soluble Fe and Cu. All of the experiments were carried out in duplicates. Bioleached residues were analyzed by X-ray diffraction and XANES analyses.

8.2.2 Overtime changes in characteristics of residual minerals during the chalcopyrite bioleaching system using mixed culture of *At. caldus* KU and *Sb. sibiricus* N1

Cells of the *At. caldus* KU and *Sb. sibiricus* N1 were pre-grown individually, harvested, washed as described above. Nonuplicate flasks of the mixed culture B (strains KU and N1), were set up to monitor overtime changes in characteristics of residual minerals during the chalcopyrite bioleaching. Bioleaching conditions of this experiment was exactly same as those of mixed culture B in 8.2.2. Samples were regularly taken to monitor cell density, pH values, solution redox potential values, and concentrations of Fe(II), total soluble Fe and Cu. Bioleached residues were applied to X-ray diffraction analysis, SEM observation, XPS analysis, EPMA, and XANES analyses.

8.3 Results and discussion

8.3.1 Effect of the microbiological control of solution redox potentials on chalcopyrite bioleaching efficiency using mixed cultures of *At. caldus* KU and either *Sb. sibiricus* N1, *Sulfobacillus* sp. YTF1, *Acidiplasma* sp. Fv-Ap, or *Am. ferrooxidans* ICP

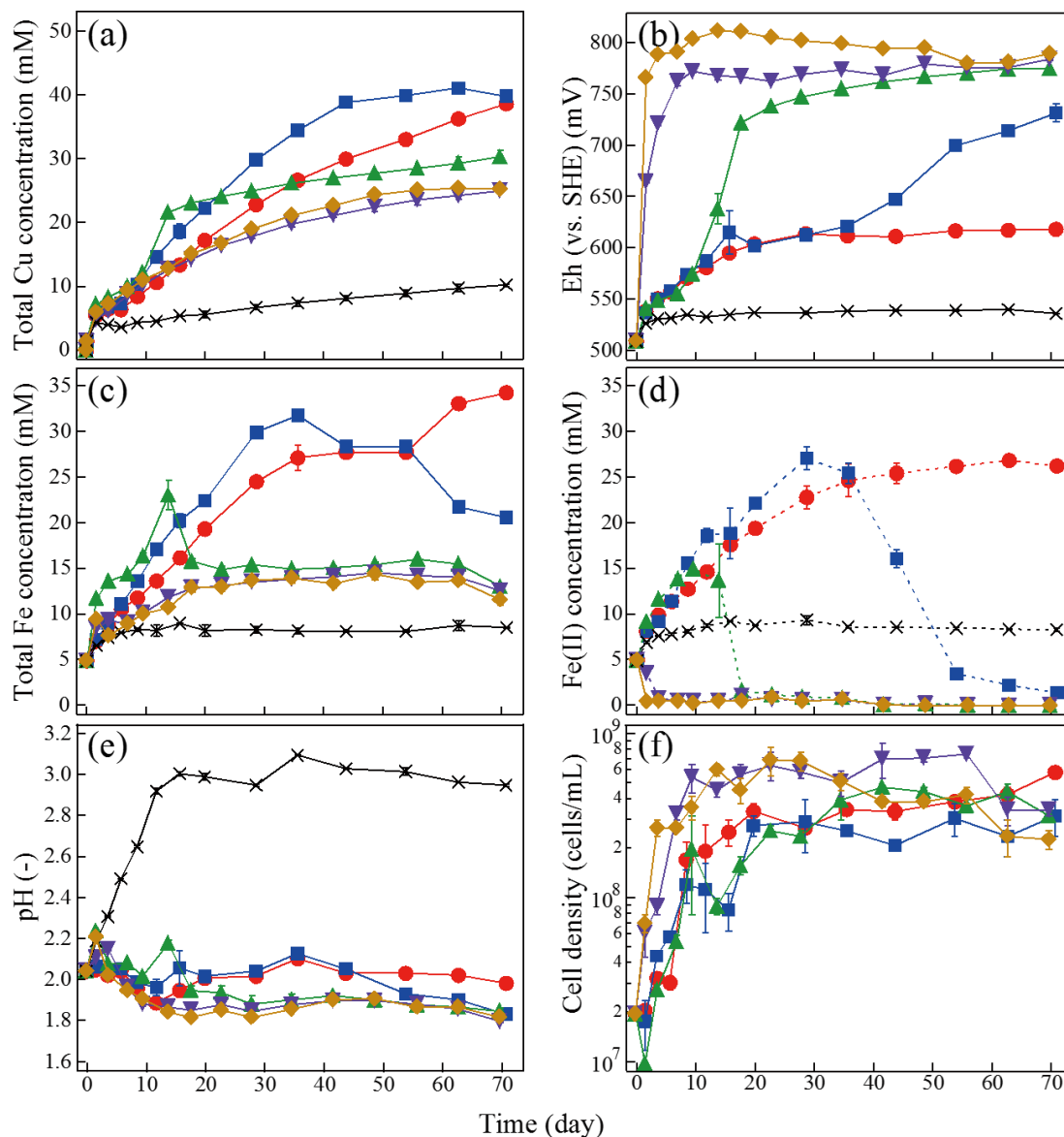


Fig. 8.1 Changes in the total Cu concentration (a), solution redox potentials (b), total Fe concentration (c), Fe(II) concentration (d), pH values (e), and cell density (f) in mixed cultures A (KU and YTF1; ●), B (KU and N1; ■), C (KU and Fv-Ap; ▲), and D (KU and Fv-Ap with yeast extract; ▼), E (KU and ICP with yeast extract; ◆) and sterile controls F (×), respectively. Data points are mean values from duplicate cultures; error bars depicting averages are not visible as these were smaller than the data point symbols in all cases.

In this experiment, five types of mixed cultures; A (KU and YTF1), B (KU and N1), C (KU and Fv-Ap), D (KU and Fv-Ap in the presence of 0.01% (w/v) y.e.), and E (KU and ICP in the presence of 0.01% (w/v) y.e.), were set up in order to investigate the effect of solution redox potentials during the chalcopyrite bioleaching test. *Sb. acidophilus* strain YTF1 and *Sb. sibiricus* strain N1, both moderately thermophilic acidophiles showing relatively weaker Fe(II)-oxidizing capability, were used in this study in order to maintain relatively low solution redox potentials ($0 < E_{\text{normal}} < 1$) during the bioleaching test. In contrast, *Acidiplasma* sp. strain Fv-Ap and *Am. ferrooxidans* strain ICP were used as a moderately thermophilic acidophiles with relatively stronger Fe(II)-oxidizing capability in order to maintain relatively high solution redox potentials ($E_{\text{normal}} > 1$). Note that *At. caldus* strain KU was added to all the mixed cultures as a moderately thermophilic S^0 -oxidizing acidophile in order to maintain pH values as low as below 2.0 and to avoid an accumulation of elemental sulfur onto the chalcopyrite surface.

In the mixed cultures A (KU and YTF1), B (KU and N1), and C (KU and Fv-Ap), microbial growth was observed during the bioleaching test (Fig. 8.1): Strain KU was the dominant species in the three mixed cultures until day 6, and subsequently, a gradual cell increase of each of Fe(II)-oxidizers was observed in mixed cultures A (KU and YTF1) and C (KU and Fv-Ap), based on the morphological difference between *At. caldus* and Fe(II)-oxidizers. This observation indicates that strain KU grew autotrophically on S^0 first, possibly followed by cell growth of heterotrophic Fe(II)-oxidizers on organic substrates deriving from cell exudates/lysates of strain KU. Microbial S^0 -oxidizing activity by strain KU resulted in culture acidification in all mixed cultures (pH values were kept between 1.8-2.2 throughout the experiment), while pH values in sterile controls increased up to 3 within 15 days (Fig. 8.1). As for mixed cultures D (KU and Fv-Ap with y.e.) and E (KU and ICP with y.e.), active cell growth was readily observed due to the initial addition of the organic substrate, resulted in a significant increase in their solution redox potentials over 700 mV within the first 4 days and being maintained at around 770-790 mV throughout the bioleaching test.

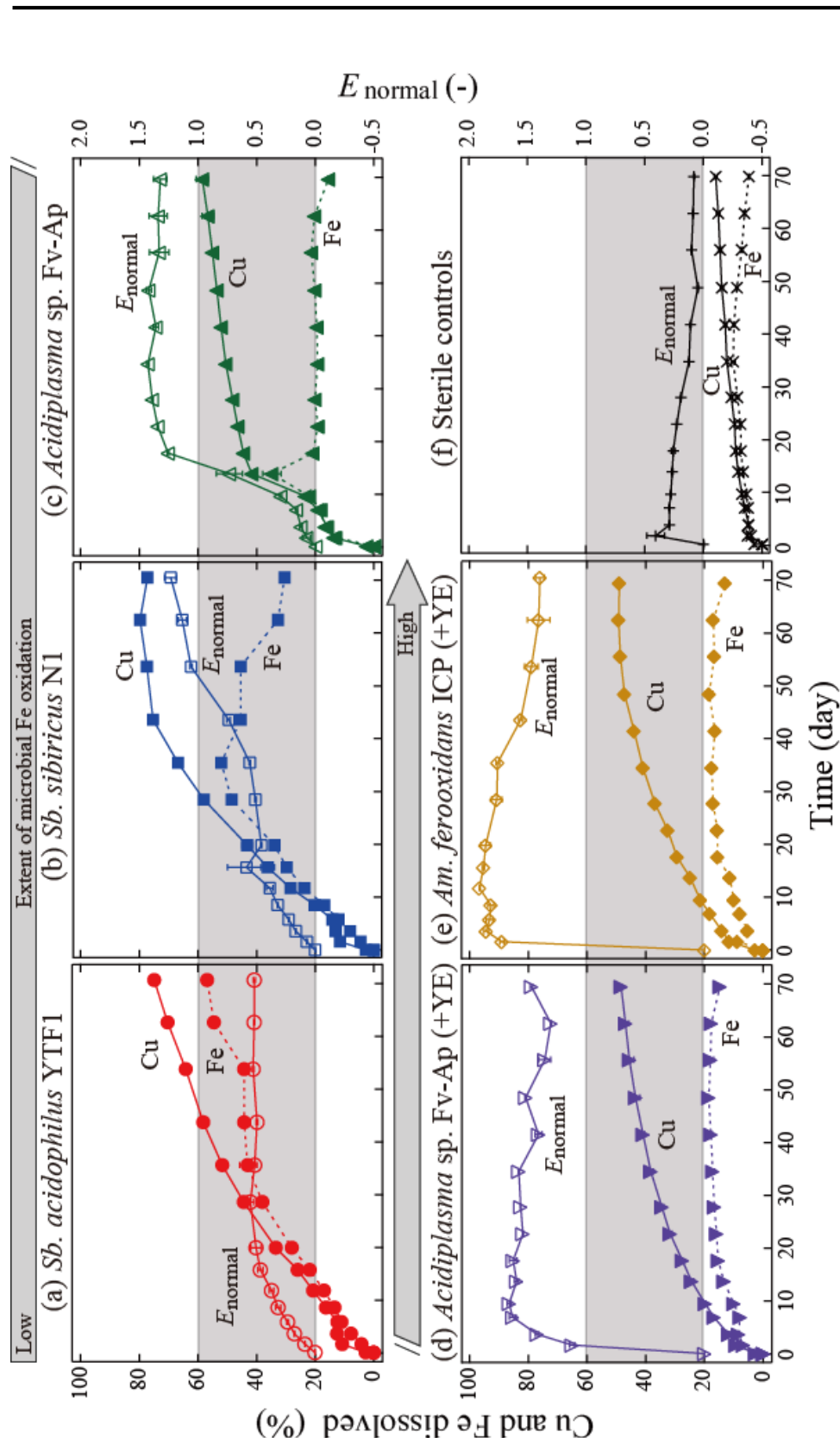


Fig. 8.2 Changes in the dissolution of Cu and Fe (left axis) and E_{normal} value (right axis) of mixed cultures A (KU and YTF1; ●), B (KU and N1; ■), C (KU and Fv-Ap; ▲), and D (KU and Fv-Ap with yeast extract; ▼), E (KU and ICP with yeast extract; ◆) and sterile controls F (×), respectively. The gray area indicates E_{normal} level between 0 to 1. Data points are mean values from duplicate cultures; error bars depicting averages are not visible as these were smaller than the data point symbols in all cases.

The Cu recovery in mixed cultures at day 70 reached 49-77%, while that in sterile controls was only 20% (Fig. 8.2). The Cu extraction behaviors in mixed cultures and even sterile controls were found to correspond to changes in the E_{normal} values. Comparing mixed cultures A (KU and YTF1) and D (KU and Fv-Ap with y.e.), the E_{normal} values in the former gradually increased up to approximately 0.5 (corresponding to approximately 620 mV of solution redox potential) until day 16, and was stably maintained throughout the bioleaching test, while that in the latter dramatically increased over 1.5 (corresponding to 760-790 mV) within the first 7 days, and also stably maintained by the end of the experiment (Fig. 8.1, 8.2). This significant difference in the E_{normal} values in the both mixed cultures; A and D, resulted in 75 and 49% Cu recovery in the former and the latter, respectively, indicating that the E_{normal} values critically affect Cu extraction efficiency in chalcopyrite bioleaching system (Fig. 8.2). Note that Cu leaching behavior and final Cu recovery in mixed cultures E (KU and ICP with y.e.; 48%) was almost similar to those in mixed cultures D (KU and Fv-Ap with y.e.; 49%).

In mixed cultures B (KU and N1), and C (KU and Fv-Ap), the E_{normal} values were within the range $0 < E_{\text{normal}} < 1$ (corresponding to $E_{\text{ox}} < E < E_{\text{c}}$) until day 44 in the former and until day 14 in the latter, respectively (Fig. 8.2). Subsequently, further increases in the E_{normal} values $1.2-1.3 < E_{\text{normal}}$ (corresponding to 730-780 mV of solution redox potential) were observed. Cu extraction in both mixed cultures; B and C, were found to slow down after the E_{normal} values exceeded over 1.0, resulting in the final Cu recovery of 77 and 59% in the former and the latter, respectively (Fig. 8.2). This finding also supports the fact that Cu extraction from chalcopyrite is strongly affected by the E_{normal} levels, and facilitated within the range $0 < E_{\text{normal}} < 1$, especially around the E_{normal} value of 0.43, as was suggested by Matsumoto et al (2004) and Hiroyoshi et al (2008).

In mixed culture C (KU and Fv-Ap), a sudden increase in total soluble Cu concentration at day 10-14 was observed, due to an increase in the E_{normal} values from approximately 0.3 to 0.7 (Fig. 8.2), resulting in the promotion of chalcocite dissolution that formed and accumulated until day 10. This leaching reaction in mixed culture C (KU and Fv-Ap) without y.e., resulted in the final Cu recovery of 59%, 10 points higher than that in mixed culture D (KU and Fv-Ap with y.e.; 49% of Cu recovery). Although

an increase in the E_{normal} values was also observed after day 44 in mixed culture B (KU and N1), its further Cu extraction was not detected. The reason for this might be an assumption that chalcocite readily dissolved without accumulation as soon as it was formed when the E_{normal} values were maintained at around 0.43. These findings imply that chalcocite dissolution is very susceptible to solution redox potentials and detectable chalcocite (actually in the form of covellite) exists only in early stage where the E_{normal} values are below at least 0.43.

The highest (77%) and the second highest (75%) Cu recoveries at day 70 were observed in mixed cultures B (KU and N1), and A (KU and YTF1), respectively (Fig. 8.2). Note that Cu recovery in the former already reached 75% at day 44. On the other hands, Cu recovery in mixed culture D (KU and Fv-Ap with y.e.) and E (KU and ICP with y.e.), reached 49 and 48%, respectively, and their Cu extraction behaviors were much slower than that in mixed cultures A and B. Therefore, effective Cu extraction from chalcopyrite was found to be achieved when the E_{normal} values were maintained in the middle level of the specific range $0 < E_{\text{normal}} < 1$. Furthermore, Cu extraction was found to slow down when the E_{normal} values exceeded 1.

Formation of potassium jarosite ($\text{KFe}_3(\text{OH})_6(\text{SO}_4)_2$) was observed in all mixed cultures and even in sterile controls, according to the XRD analysis of bioleached residues (Fig. 8.3) Many researchers reported the formation of ferric precipitates, mainly jarosite, as a passivation layer on chalcopyrite surface especially at relatively high solution redox potential levels, in both bio- and chemical-leaching systems of chalcopyrite (Córdoba et al 2008, Fu et al 2008, Zhu et al 2011, Sasaki et al 2009, Sandström et al 2005). Larger amount of jarosite might be formed in mixed cultures B, C, D, and E compared with that in mixed culture A and sterile controls F, due to higher solution redox potential levels in the formers at the end of the experiment.

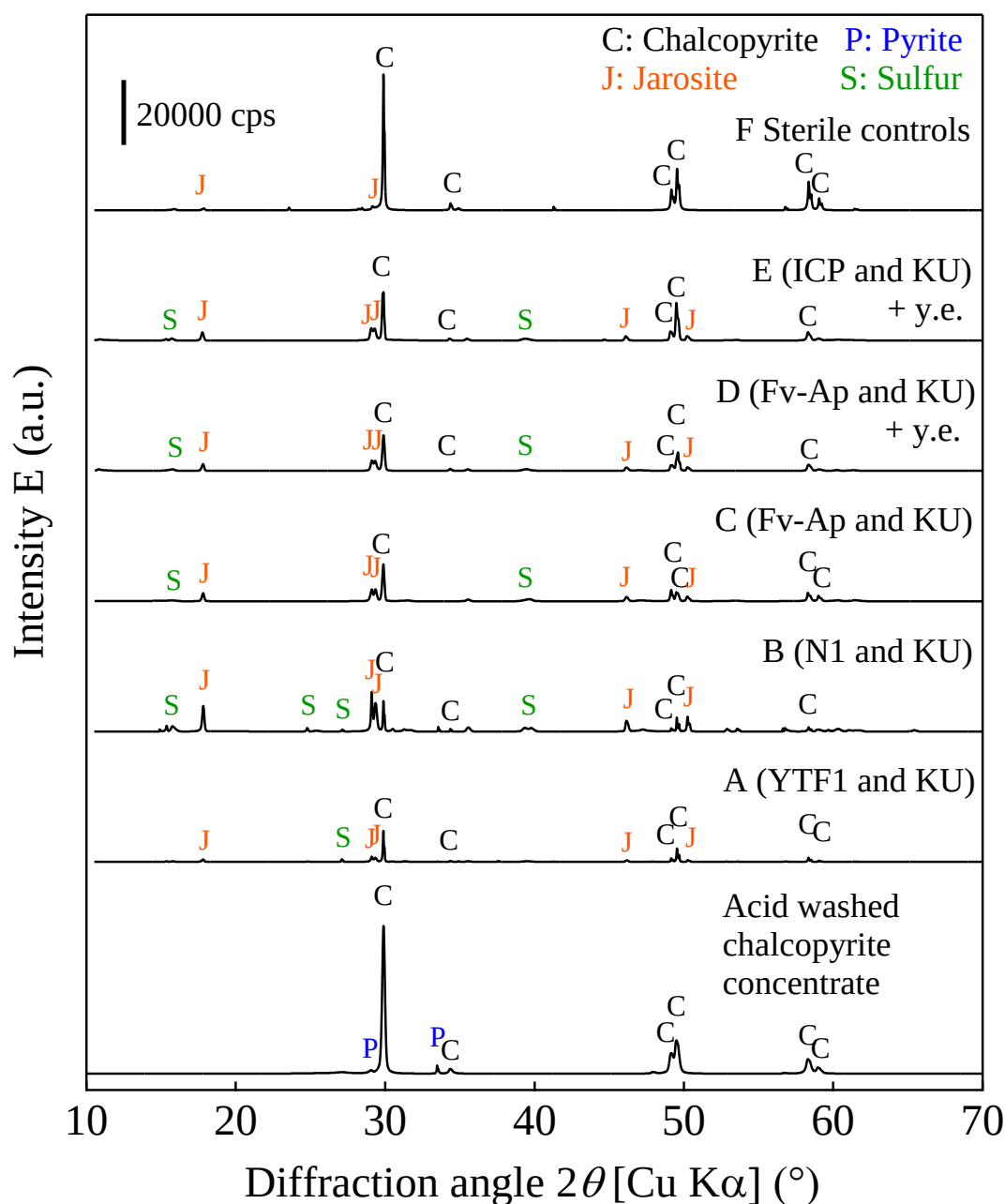
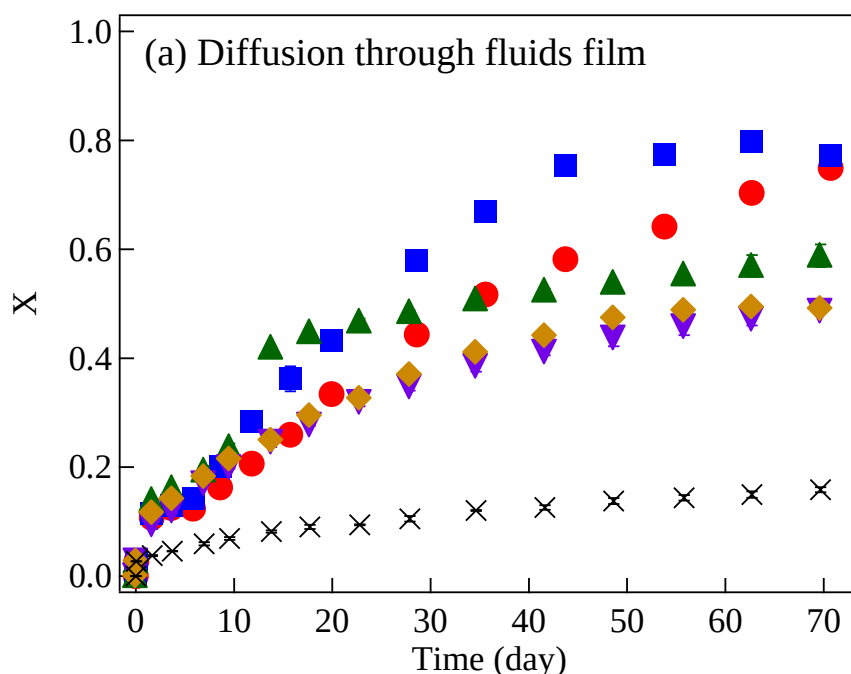


Fig. 8.3 X-ray diffraction patterns of original chalcopyrite concentrate, bioleached residues of mixed cultures A (strains YTF1 and KU), B (strains N1 and KU), C (strains Fv-Ap and KU), and D (strains Fv-Ap and KU with the addition of yeast extract) and leached residue of sterile controls E, from the bottom to the top. C; chalcopyrite (NIST AL2358), P; pyrite (COD 9000595), J; potassium jarosite (ICSD 166801), S; elemental sulfur (ICSD 412326).

~ Kinetic study ~

Using the final Cu recovery values in the previous experiment as X ($0 < X < 1$), if a function of X in either the rate equation Eq. 8.7-8.9 shows a linear correlation to leaching time, it is indicated that the dissolution kinetic of chalcopyrite in the previous bioleaching test is controlled by diffusion through fluid film, surface chemical reaction, or diffusion through solid product film. Note that the leaching period after day 2 was used for the calculations in all mixed cultures and sterile controls since Cu recover in the first 2 days unlikely originated from chalcopyrite, probably deriving from other more amenable Cu-containing minerals associated with chalcopyrite concentrate used in this study (leached Cu concentration at day 2 was the same level among all mixed cultures and sterile controls). In mixed cultures B (KU and N1) and C (KU and Fv-Ap), two approximate straight lines were tried to draw for each due to their transitions in E_{normal} levels. Only for the latter part (day 54-71) of mixed cultures B, drawing the line was, however, avoided since there were only three data points left.



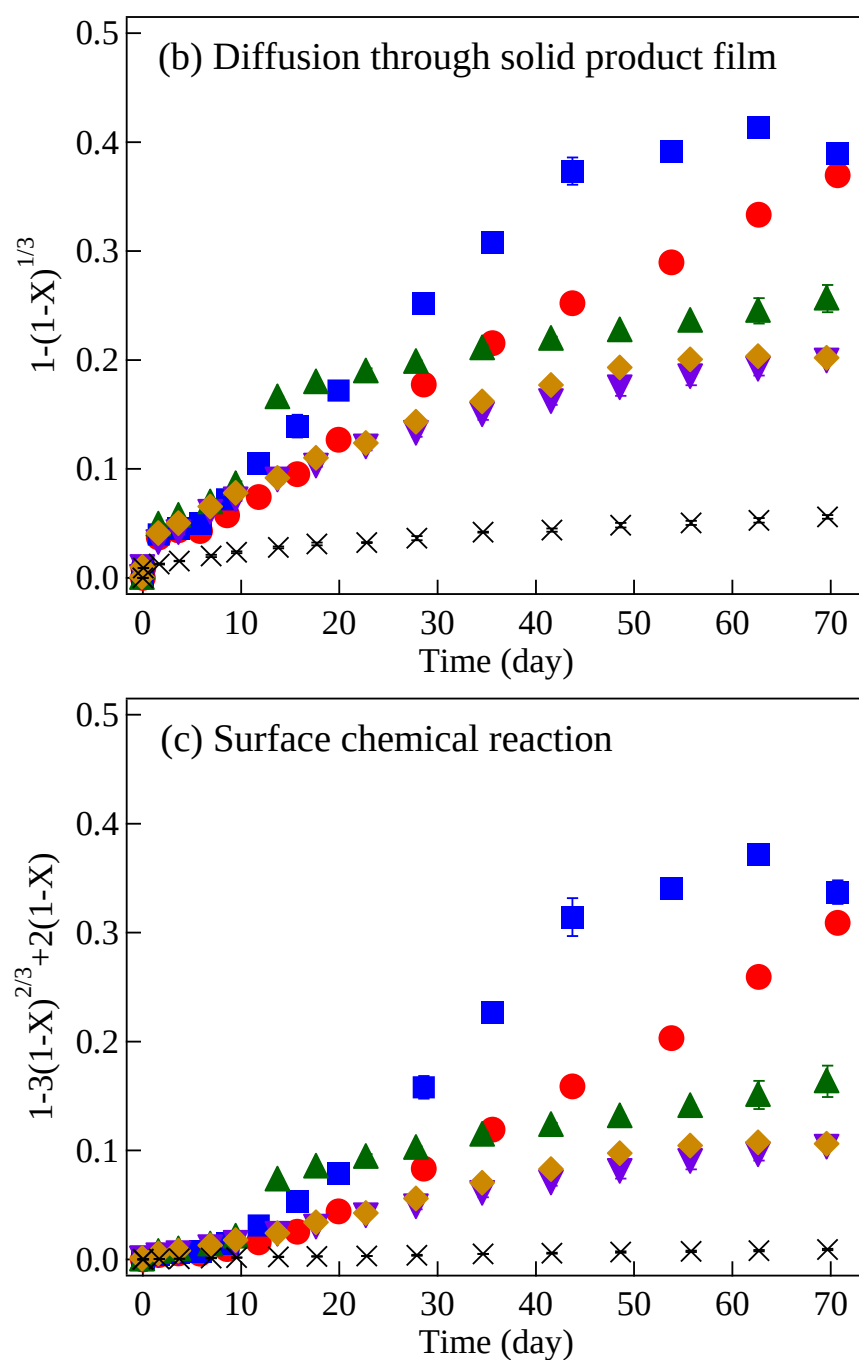


Fig. 8.4 Plots of (a) Eq. 8.7 (diffusion through fluid film), (b) Eq. 8.8 (diffusion through solid product film), and (c) Eq. 8.9 (surface chemical reaction) vs. leaching time for mixed cultures A (KU and YTF1; ●), B (KU and N1; ■), C (KU and Fv-Ap; ▲), and D (KU and Fv-Ap with yeast extract; ▼), E (KU and ICP with yeast extract; ◆) and sterile controls F (×). Data points are mean values from duplicate cultures; error bars depicting averages are not visible as these were smaller than the data point symbols in all cases.

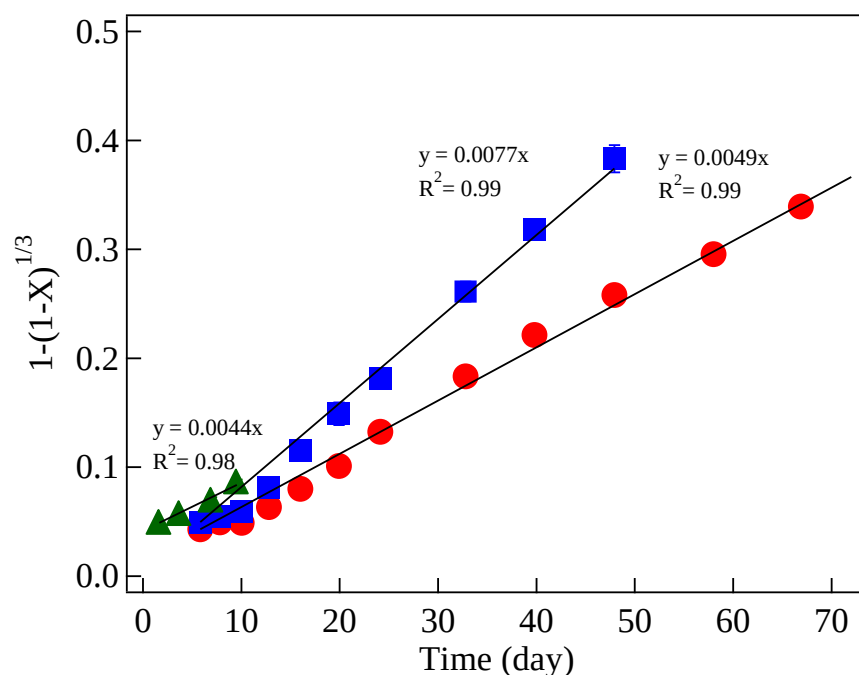


Fig. 8.5 Fitting plots of Eq. 8.9 (surface chemical reaction) vs. leaching time for mixed cultures A (strains YTF1 and KU; ●), B (strains N1 and KU; ■), and C (strains Fv-Ap and KU; ▲).

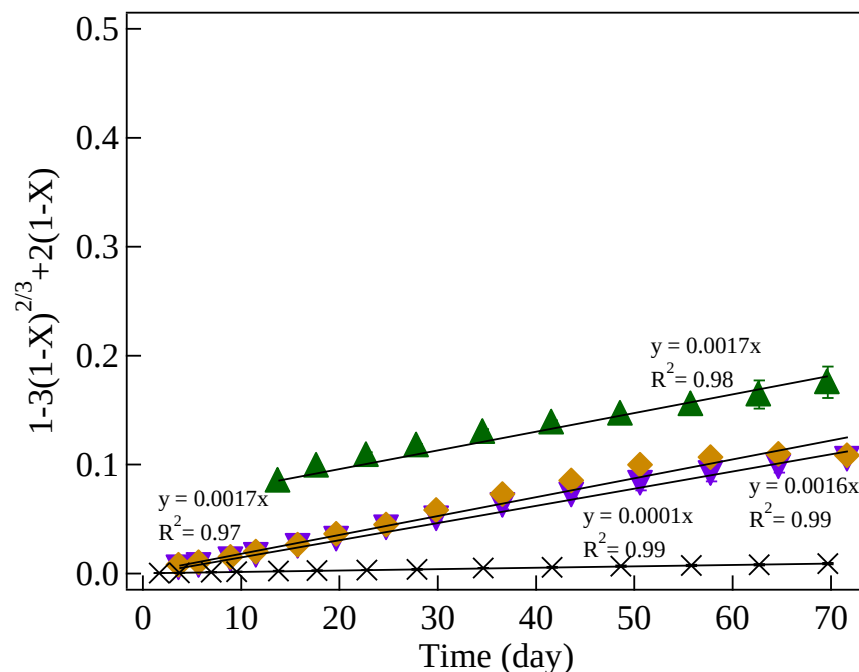


Fig. 8.6 Fitting plots of Eq. 8.8 (diffusion through solid product film) vs. leaching time for mixed cultures C (strains Fv-Ap and KU; ▲), D (strains Fv-Ap and KU with the addition of yeast extract; ▼), and E (KU and ICP with yeast extract; ◆), and sterile controls F (×).

Linear correlations against surface chemical reaction control model given in Eq. 7 were shown in mixed cultures A (KU and YTF1; day 2-71, $R^2=0.99$), B (KU and N1; day 2-44, $R^2=0.99$), and C (KU and Fv-Ap; day 2-10, $R^2=0.98$). Linear correlations against diffusion through solid product film (Eq. 8) were found in mixed cultures C (KU and Fv-Ap; day 14-70, $R^2=0.98$), D (KU and Fv-Ap + YE; day 2-70, $R^2=0.99$) and E (KU and ICP + YE; day 2-70, $R^2=0.97$), and sterile controls (day 2-70, $R^2=0.99$). In mixed cultures, but not in sterile controls, linear correlations to surface chemical reaction or diffusion through solid product film were found at where $1 \geq E_{\text{normal}} \geq 0$ or $E_{\text{normal}} > 1$, respectively. These results imply that solid product film is likely jarosite which was restrained being formed at $1 \geq E_{\text{normal}} \geq 0$, but accumulated on chalcopyrite surface at $1 \geq E_{\text{normal}} \geq 0$. Interestingly, transition of E_{normal} level and chalcopyrite dissolution model were observed in mixed cultures C (KU and Fv-Ap): Its chalcopyrite dissolution behavior was found to be controlled by surface chemical reaction during day 2-10 when its E_{normal} level was maintained at $1 \geq E_{\text{normal}} \geq 0$. After day 14, its dissolution model was found to turn into diffusion through solid product film (day 14-70) due to higher E_{normal} level over 1.

Unexpectedly, chalcopyrite dissolution in sterile controls was found to be controlled by diffusion through solid product film, but not by surface chemical reaction, although its E_{normal} level was maintained at the bottom of the specific range between 0 to 1, implying the formation of solid product film on chalcopyrite surface even in sterile controls. Due to the increasing pHs in sterile controls (reaching 2.9 at day 70), jarosite formation on the mineral surface is likely. Very thin jarosite layers on the mineral surface in bioleached residues of sterile controls were observed by EPMA as shown in Fig. 8.7.

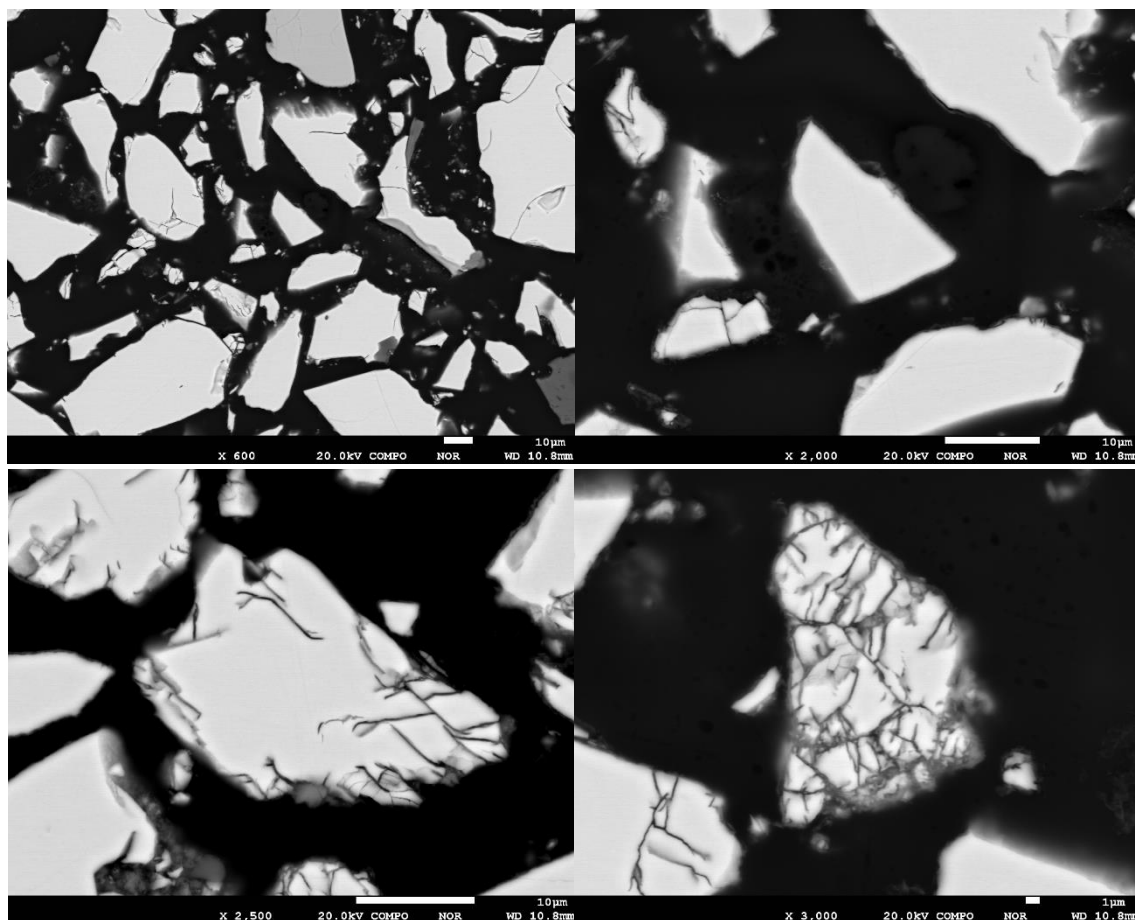


Fig. 8.7 EPMA observation of leached residues of sterile controls F (in the presence of yeast extract).

The k_r and k_d values of the previous chalcopyrite bioleaching test which were obtained via fitting plots on models of surface chemical reaction control and diffusion through solid product film control, respectively, are summarized in Table 8.1. The k_r values in mixed cultures A (day 2-71), B (day 2-44) and C (day 2-10) were much higher than the k_d values in mixed cultures C (day 14-70), D (day 2-70) and E (day 2-70). The k_d value; 0.0001, obtained in sterile controls (day 2-70) was the lowest among them. Therefore, chalcopyrite dissolution controlled by the former model was faster than that by the latter. Overall, chalcopyrite dissolution at $1 \geq E_{\text{normal}} \geq 0$ likely occurred through chalcocite formation and its oxidation, being controlled by surface chemical reaction, while that at $E_{\text{normal}} > 1$ was likely based on oxidation by Fe(III) (due to its higher concentration than that at $1 \geq E_{\text{normal}} \geq 0$), being controlled by diffusion through solid product film (jarosite).

Table 8.1 Summary of kinetic study of chalcopyrite dissolution in the bioleaching systems.

Mixed cultures and sterile controls	Additives	Fe-oxidizing microorganisms	Leaching periods (day)	Surface chemical reaction		Diffusion through solid product film	
				k_r	R^2	k_d	R^2
A (●)	-	<i>Sb. acidophilus</i> YTF1	2 - 71	0.0049	0.99	0.0039	0.95
B (■)	-	<i>Sb. sibiricus</i> N1	2 - 44	0.0077	0.99	0.0062	0.92
			54 - 71	-	-	-	-
C (▲)	-	<i>Acidiplasma</i> sp. Fv-Ap	2 - 10	0.0044	0.98	0.0016	0.95
			14 - 70	0.0017	0.95	0.0017	0.98
D (▼)	0.01% YE	<i>Acidiplasma</i> sp. Fv-Ap	2 - 70	0.0029	0.89	0.0016	0.99
E (◆)	0.01% YE	<i>Am. ferrooxidans</i> ICP	2 - 70	0.0030	0.89	0.0017	0.97
F (×)	0.01% YE	Cell free	2 - 70	0.0007	0.90	0.0001	0.99

Fig. 8.8 shows the plots of Cu extraction rate (with a unit of mM/day) vs. the E_{normal} values of five mixed cultures A, B, C, D and E, and sterile controls. Enhancement of Cu extraction rate was clearly seen to be maximized at E_{normal} value of approximately 0.3. The trend in the Cu extraction rate vs. E_{normal} values is very similar to that reported by Okamoto et al. (2004) and Hiroyoshi et al. (2008): Highest Cu extraction rate was reported at E_{normal} value of 0.43. Therefore, dissolution efficiency of chalcopyrite via surface chemical reaction control model with E_{normal} values of around 0.3, was indicated to be much more efficient compared with that via diffusion through solid product film. This has contributed to the variation of Cu recovery in mixed cultures; A, B, C, D and E, differently controlled by their solution redox potentials owing to different degrees of microbial Fe-oxidizing capability.

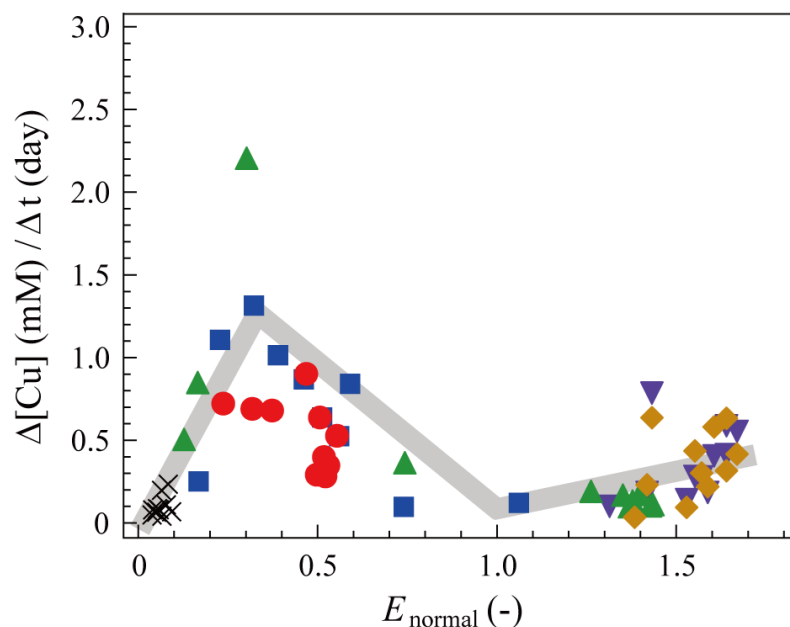


Fig. 8.8 Plots of Cu recovery rate vs. E_{normal} values in mixed cultures A (KU and YTF1; ●), B (KU and N1; ■), C (KU and Fv-Ap; ▲), and D (KU and Fv-Ap with yeast extract; ▼), E (KU and ICP with yeast extract; ◆) and sterile controls F (×).

8.3.2 Overtime changes in characteristics of residual minerals during the chalcopyrite bioleaching system using mixed culture of *At. caldus* KU and *Sb. sibiricus* N1

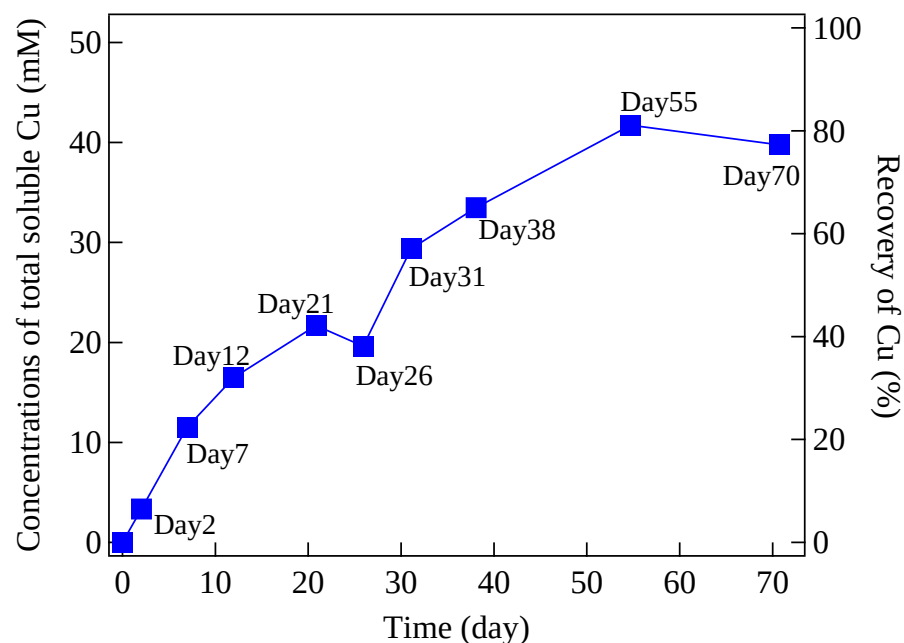


Fig. 8.9 Changes in the concentration of total soluble Cu in mixed culture B (strains KU and N1).

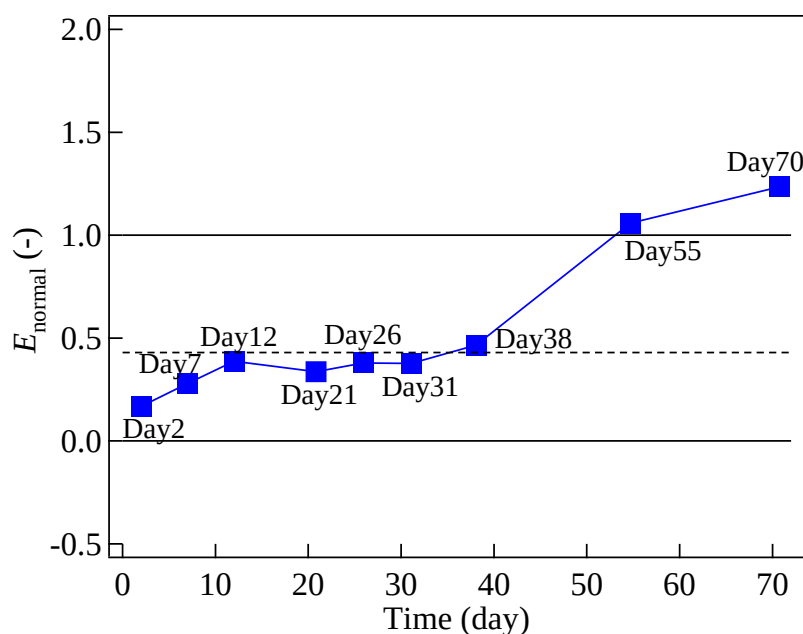


Fig. 8.10 Changes in the E_{normal} value in mixed culture B (strains KU and N1).

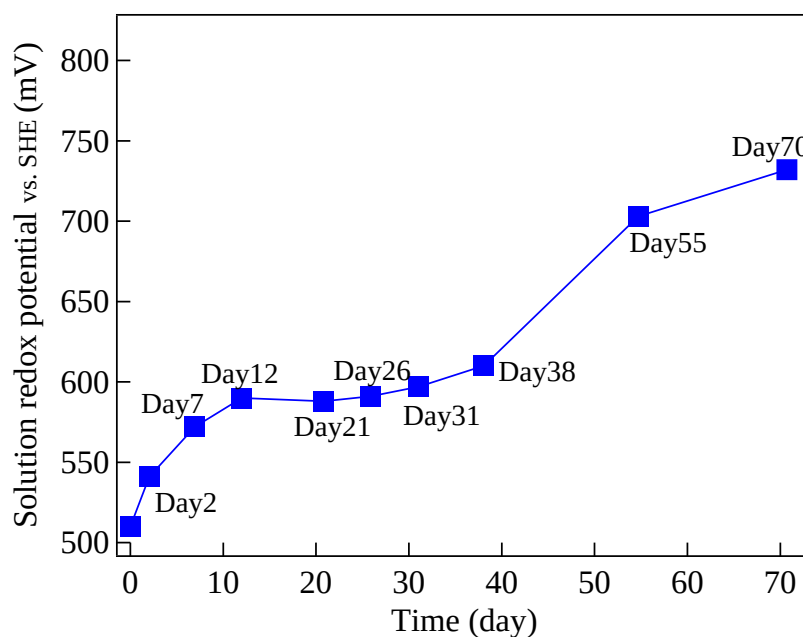


Fig. 8.11 Changes in the solution redox potentials in mixed culture B (strains KU and N1).

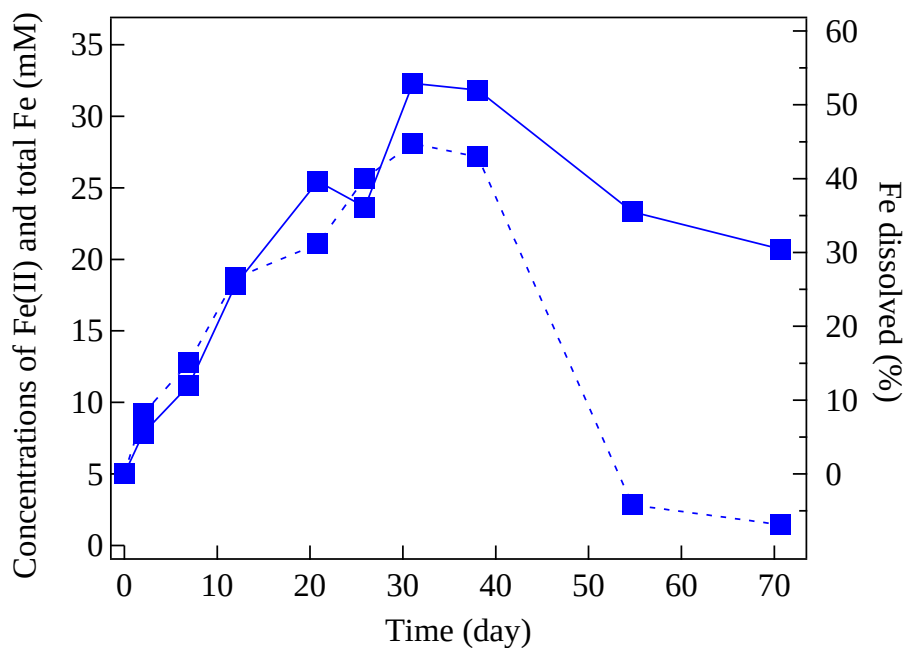


Fig. 8.12 Changes in the concentrations of total soluble Fe (solid lines) and Fe(II) (broken lines) in mixed culture B (strains KU and N1).

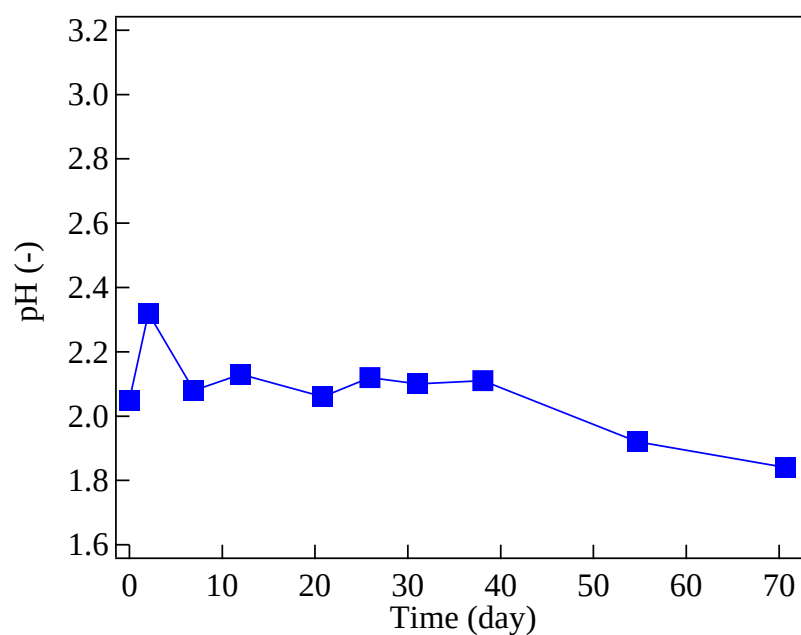


Fig. 8.13 Changes in the pHs in mixed culture B (strains KU and N1).

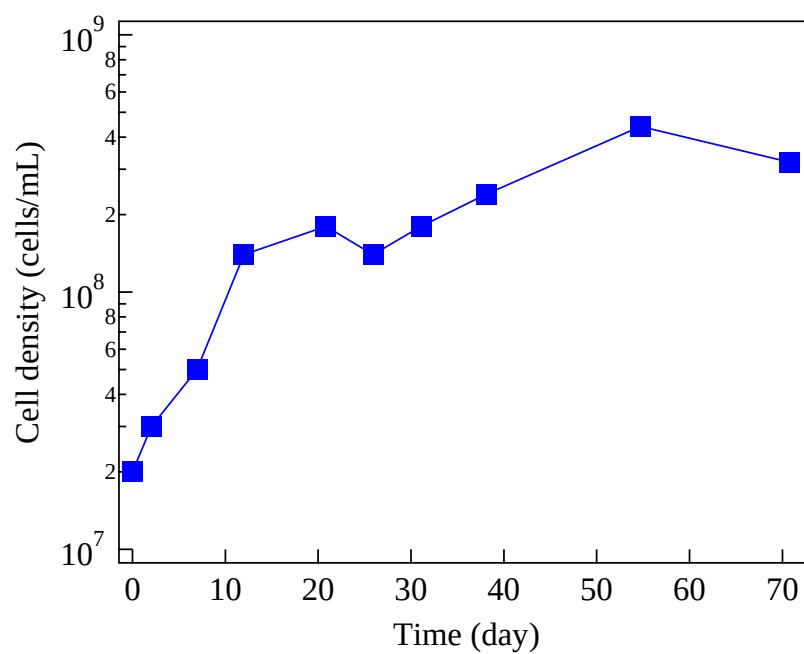


Fig. 8.14 Changes in the cell density in mixed culture B (strains KU and N1).

The changes in Cu concentrations and E_{normal} values in mixed culture B (KU and N1), were shown in Fig. 8.10. X-ray diffraction analysis showed the formation of jarosite and also accumulation of elemental sulfur in the bioleached residues only after day 38, but not before the day (Fig. 8.15). Peaks of Cu 2p_{3/2}, Cu 2p_{1/2}, S 2p_{3/2}, and S 2p_{1/2} deriving from chalcopyrite were shown in XP-spectra of bioleached residues until day 31 (i.e., the Cu 2p_{3/2} and Cu 2p_{1/2} peaks reported to be at 932.4 and 952.3, respectively (Ghahremaninezhad et al. 2013), the Cu 2p_{3/2} peak reported to be at 931.7 eV (Sasaki et al. 2010), 932.2 eV (Mielczarski et al. 1996), the S 2p_{3/2} and S 2p_{1/2} peaks (monosulfide) reported to be at 161.4 and 162.6 eV (Ghahremaninezhad et al. 2013), 160.8 and 162.0 eV (Sasaki et al. 2010), 161.2 and 162.3 eV (Klauber et al. 2001), respectively). Note that (i) XP-spectra of S 2p deriving from chalcopyrite are involved by monosulfide, disulfide, polysulfide, elemental sulfur and sulfate (Klauber et al. 2001, Ghahremaninezhad et al. 2013), and (ii) that small peaks associating with the main peaks of Cu 2p_{3/2} and Cu 2p_{1/2} at a few higher binding energies are likely due to differential charging effect. In addition, a S 2p peak at 172.5 eV in bioleached residue (day 31) likely originated from sulfate in jarosite since it is likely shifted due to also differential charging effect (electric conductivity of jarosite and chalcopyrite is different). Disappearance of Cu 2p peaks (deriving from chalcopyrite) and appearance of S 2p peaks (deriving from sulfate; its S 2p_{3/2} and S 2p_{1/2} peaks reported to be at 168.7 and 169.9 eV, respectively (Klauber et al. 2001)) in bioleached residues after day 38 indicate that chalcopyrite surface was covered by secondary-formed jarosite as passivation layers, since XPS analysis is specifically sensitive to surface of samples. This jarosite formation on chalcopyrite surface after day 38 was also supported by color changes in bioleached residues: The color of bioleached residues was found to become yellow from black (original color of chalcopyrite concentrate). The color transition was clearly seen in bioleached residues after day 31. Therefore, the slowing down in Cu recovery rate in mixed cultures B after day 38 can be explained by changes in chalcopyrite dissolution model: Its dissolution was controlled by surface reaction until day 38, and subsequently turned into being controlled by diffusion through jarosite layers, with the faster Cu dissolution rate in the former than the latter.

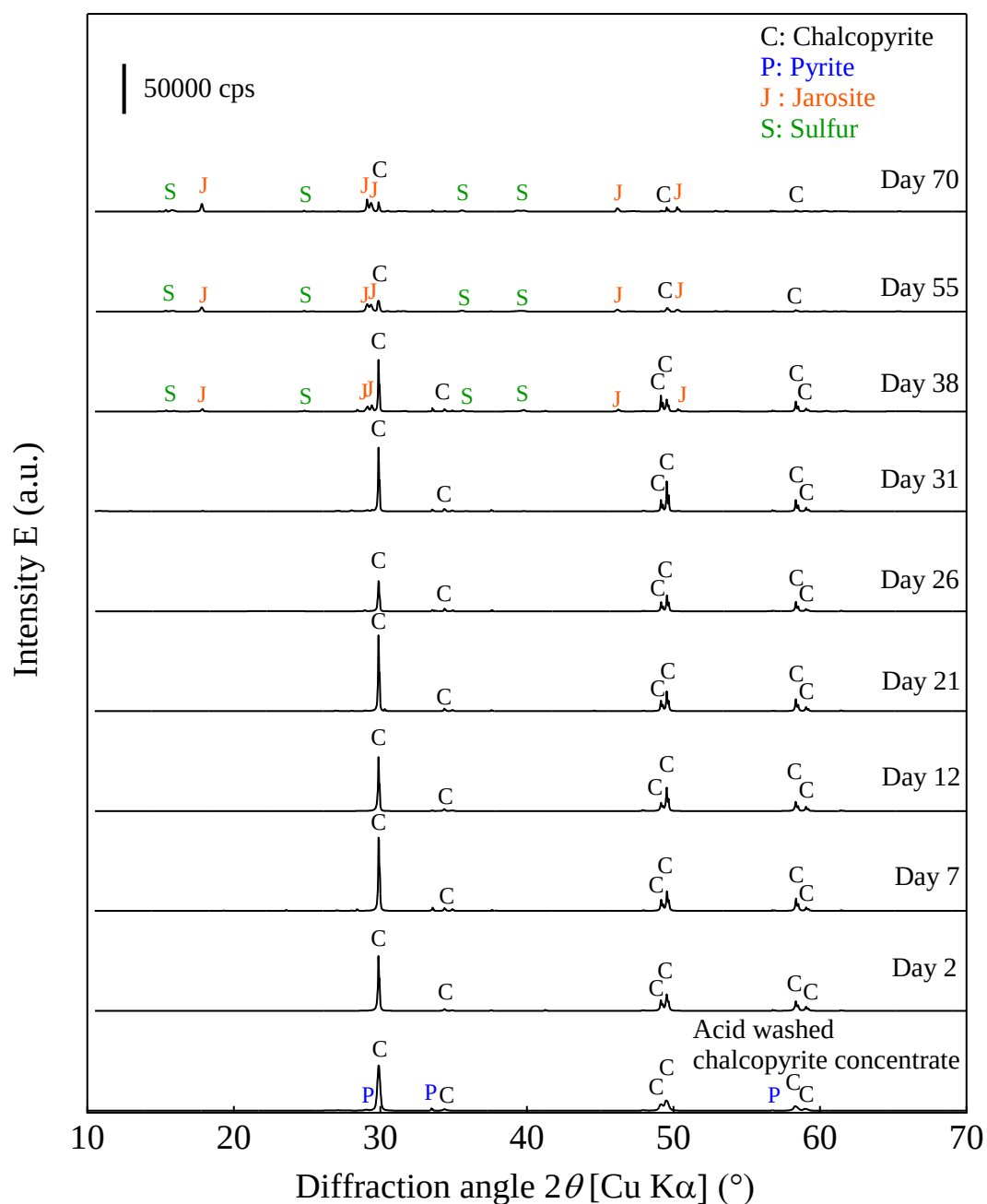


Fig. 8.15 X-ray diffraction patterns of original chalcopyrite concentrate and bioleached residues of mixed cultures B (strains N1 and KU) collected at day 2, 7, 12, 21, 26, 31, 38, 55, and 70, from the bottom to the top. C; chalcopyrite (NIST AL2358), P; pyrite (COD 9000595), J; potassium jarosite (ICSD 166801), S; elemental sulfur (ICSD 412326).

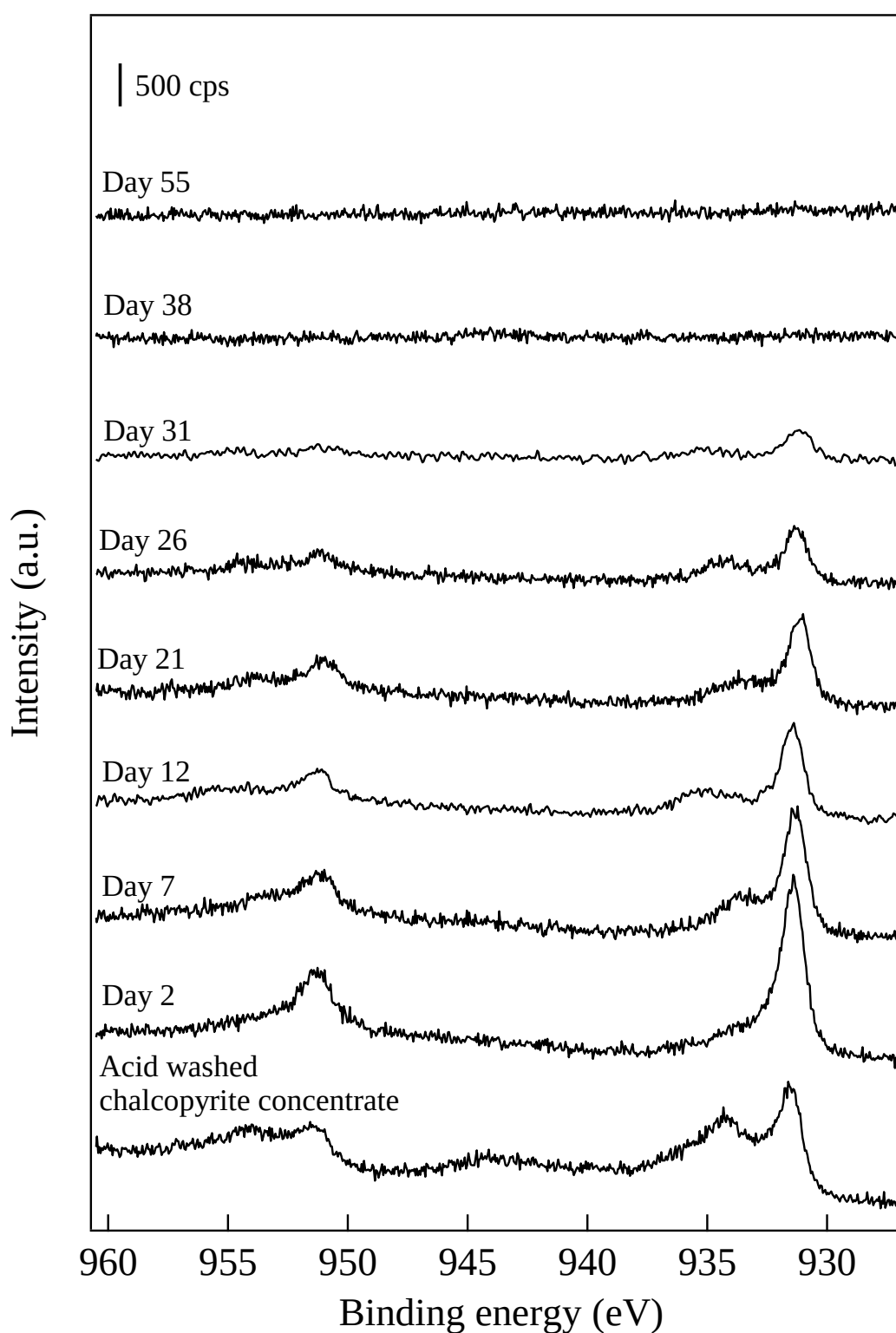


Fig. 8.16 XP-spectra of Cu 2p of original chalcopyrite concentrate and bioleached residues of mixed cultures B (strains N1 and KU) collected at day 2, 7, 12, 21, 26, 31, 38, and 55, from the bottom to the top.

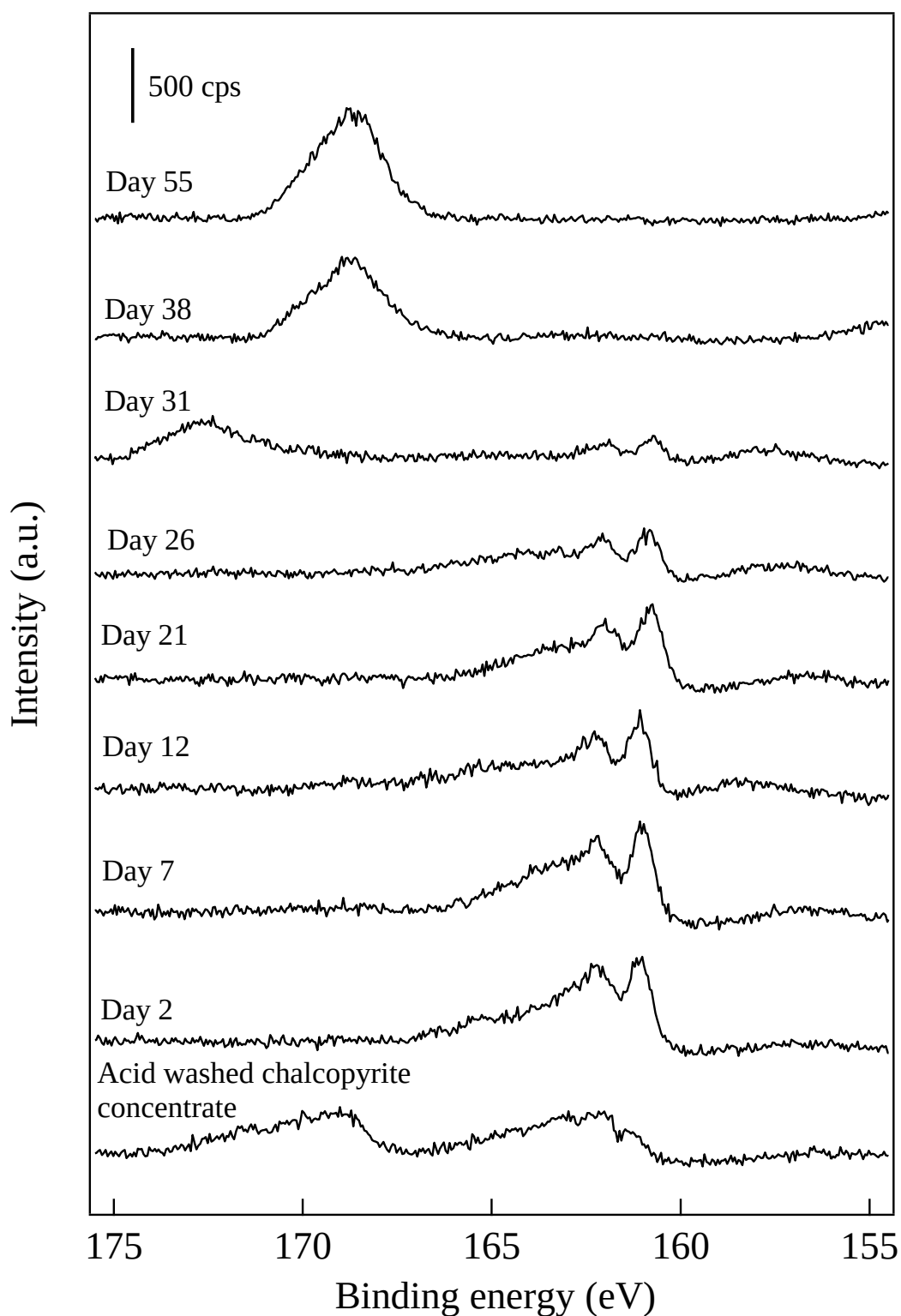


Fig. 8.17 XP-spectra of S 2p of original chalcopyrite concentrate and bioleached residues of mixed cultures B (strains N1 and KU) collected at day 2, 7, 12, 21, 26, 31, 38, and 55, from the bottom to the top.

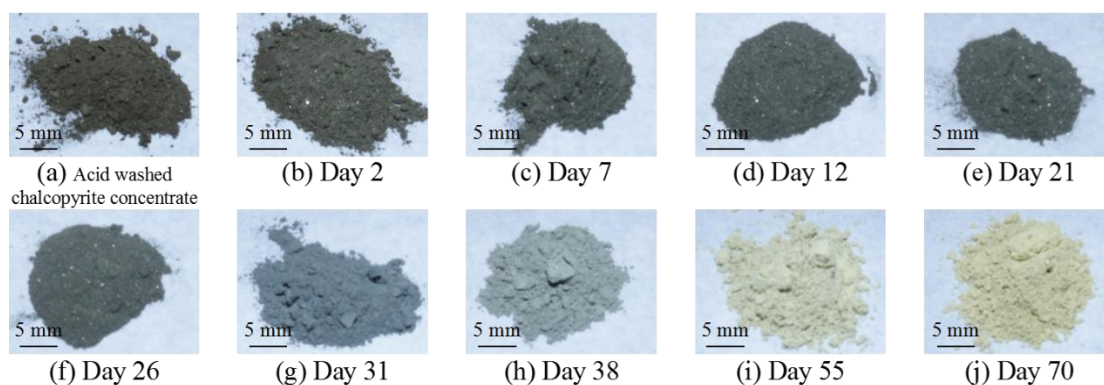


Fig. 8.18 Picture images of original chalcopyrite concentrate and bioleached residues of mixed cultures B (strains N1 and KU) collected at different leaching periods.

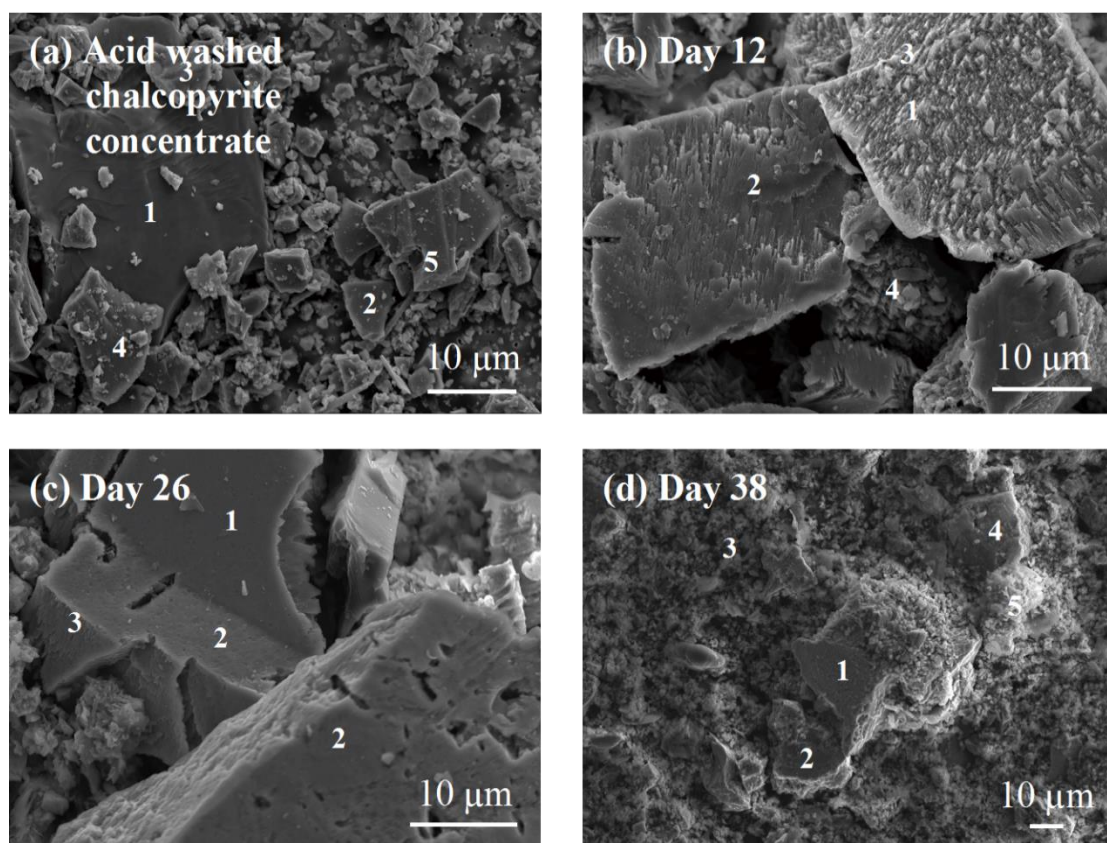


Fig. 8.19 SEM images of original chalcopyrite concentrate and bioleached residues of mixed cultures B (strains N1 and KU) collected at day 12, 26, and 38. Numbers in white color (1-5) in the images indicate beam spot positions analyzed by SEM-EDX. (a) 1-4; chalcopyrite, 5; pyrite. (b) 1-3; chalcopyrite, 4; pyrite. (c) 1-4; chalcopyrite. (d) 1; chalcopyrite, 2; pyrite, 3; potassium jarosite.

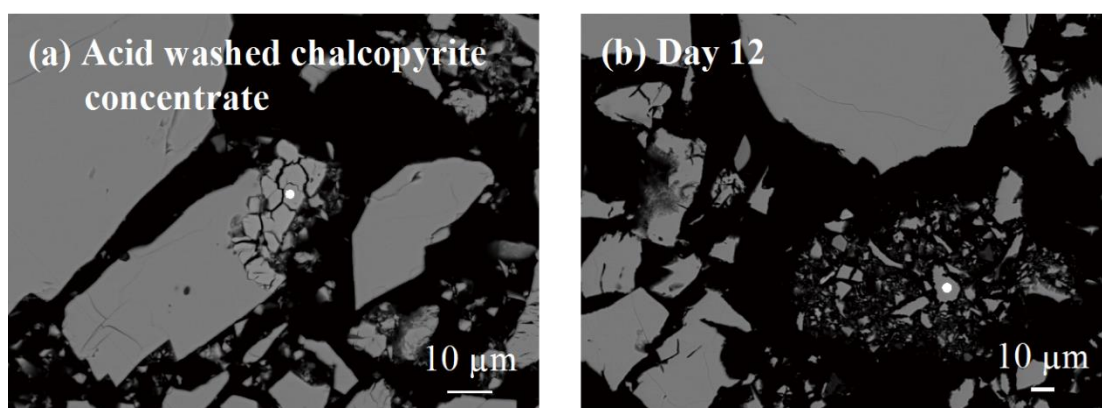
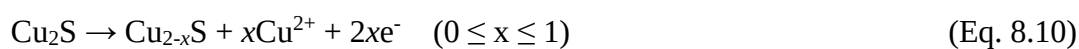


Fig. 8.20 EPMA observation (cross section images) of original chalcopyrite concentrate and bioleached residue of mixed cultures B (strains N1 and KU) collected at day 12. The white points in the images indicate the mineral particles and beam spot positions on the targets, respectively (see Table 8.2).

Table 8.2 Elemental composition of covellite-like particles in original chalcopyrite concentrate and bioleached residue at day 12 by EPMA measurement.

Samples	Elemental composition of covellite-like particles (Weight-based)						
	Cu	S	Fe	As	Pb	Co	Cu/S
Original chalcopyrite concentrate	66.4	30.4	2.4	0.0060	N.D.	N.D.	2.2
Bioleached residue collected at day 12	61.7	31.8	0.82	N.D.	N.D.	N.D.	1.9

As was described above, involvement of chalcocite into the chalcopyrite dissolution has been recognized as an important mechanism. In fact, chalcocite is readily transferred into djurjerite ($\text{Cu}_{1.93-1.96}\text{S}$), subsequently into digenite ($\text{Cu}_{1.80}\text{S}$) and finally into covellite (Córdoba et al 2008). Hiroyoshi et al (2004) reported that oxidation of chalcocite at acidic pHs proceeds as follows;



The presence of covellite in bio/chemical leached residues during the chalcopyrite dissolution was reported via Raman spectra (Sasaki et al 2009) and XRD patterns (Córdoba et al 2008). Partial contribution of both chalcocite and covellite to XANES (X-ray absorption near-edge structure) spectra of S K-edge for bioleached residues was reported (Zhu et al 2011).

As an additional solid analysis, cross section images of the original chalcopyrite concentrate and its bioleached residues at day 12 were observed by EPMA analysis: The presence of “covellite-like cracked particles” was detected in bioleached residue at day 12, however it was also observed in the original chalcopyrite concentrate (Fig. 8.21). Its elemental composition and weight based Cu/S ratio was very similar to that of pure covellite (Table 8.2). Therefore, covellite found in bioleached residue at day 12 was not necessarily formed through dissolution of chalcopyrite in this experiment.

Although accumulation of covellite, but not chalcocite, has been recognized in chalcopyrite bioleaching system at relatively low solution redox potentials, and covellite dissolution is slower than that of chalcocite (Gupta 2003), no accumulation of intermediate covellite might occur in the bioleaching systems. The reasons for this may be (i) continuous generation of Fe(III) through microbial Fe(II)-oxidizing capabilities (covellite is oxidized by Fe(III) via indirect microbial oxidation), and (ii) elemental sulfur, by-product of covellite oxidation, is oxidized by S^0 -oxidizing capability of *At. caldus* KU. Molar ratio between Cu leached and Fe leached (excluding the initial addition of 5 mM Fe(II)) was stably maintained over 1 in all mixed cultures tested throughout the bioleaching experiment (Fig. 8.21), implying that covellite formed was readily converted to soluble Cu ions without its detectable accumulation.

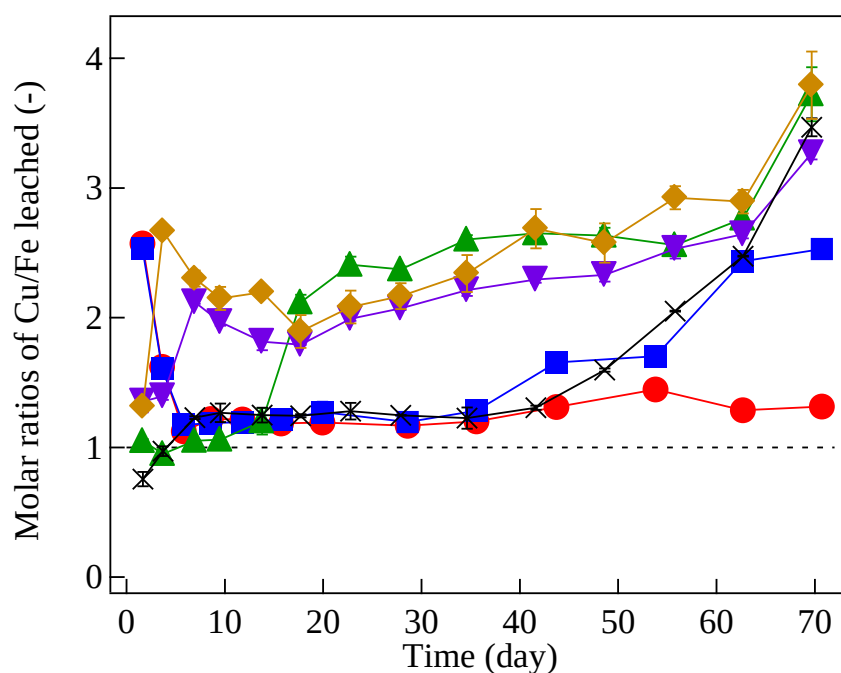


Fig. 8.21 Changes in the molar ratio of Cu/Fe leached in mixed cultures A (strains YTF1 and KU; ●), B (strains N1 and KU; ■), C (strains Fv-Ap and KU; ▼), D (strains Fv-Ap and KU with the addition of yeast extract; ▼), and E (KU and ICP with yeast extract; ◆) and sterile controls F (×). Broken line indicates a molar ratio of Cu/Fe leached of 1.0. Data points are mean values from duplicate cultures; error bars depicting averages are not visible as these were smaller than the data point symbols in all cases.

8.3.3 Comparison of XANES spectra of Cu K-edge of reference samples and bioleached residues in the previous experiments

Acid washed concentrates of chalcopyrite, chalcocite, and covellite were used as reference samples. Note that chalcopyrite concentrate was found to contain covellite (EPMA results). Fig. 8.21 shows XANES spectra of bioleached residues (at day 70) of mixed cultures A (KU + YTF1), B (KU + N1), C (KU + Fv-Ap), and D (KU + Fv-Ap, YE), and sterile controls F. All the spectra of bioleached residues were found to shift to lower energy side, compared with the spectrum of acid washed chalcopyrite concentrate. As for overtime changes in XANES spectra of bioleached residues in the previous experiment (8.3.2), the E_0 values (defined as energy values at absorption coefficient $\mu_t=0.5$) were found to gradually decrease as bioleaching proceeded (Fig. 8.23, 24, Table 8.4). This observation implies that oxidation state of Cu in chalcopyrite structure varied and that the structure itself changed. Although formation of chalcocite (actually accumulated as covellite to some extent) was thought to likely occur during the chalcopyrite bioleaching where E_{normal} values were below 1.0, accumulation of chalcocite or covellite was not shown in XANES spectra of bioleached residues collected at early stage of the experiment. Since present analyses might not be enough for the evidence of chalcocite formation as an intermediate during the chalcopyrite dissolution, further analyses would be necessary.

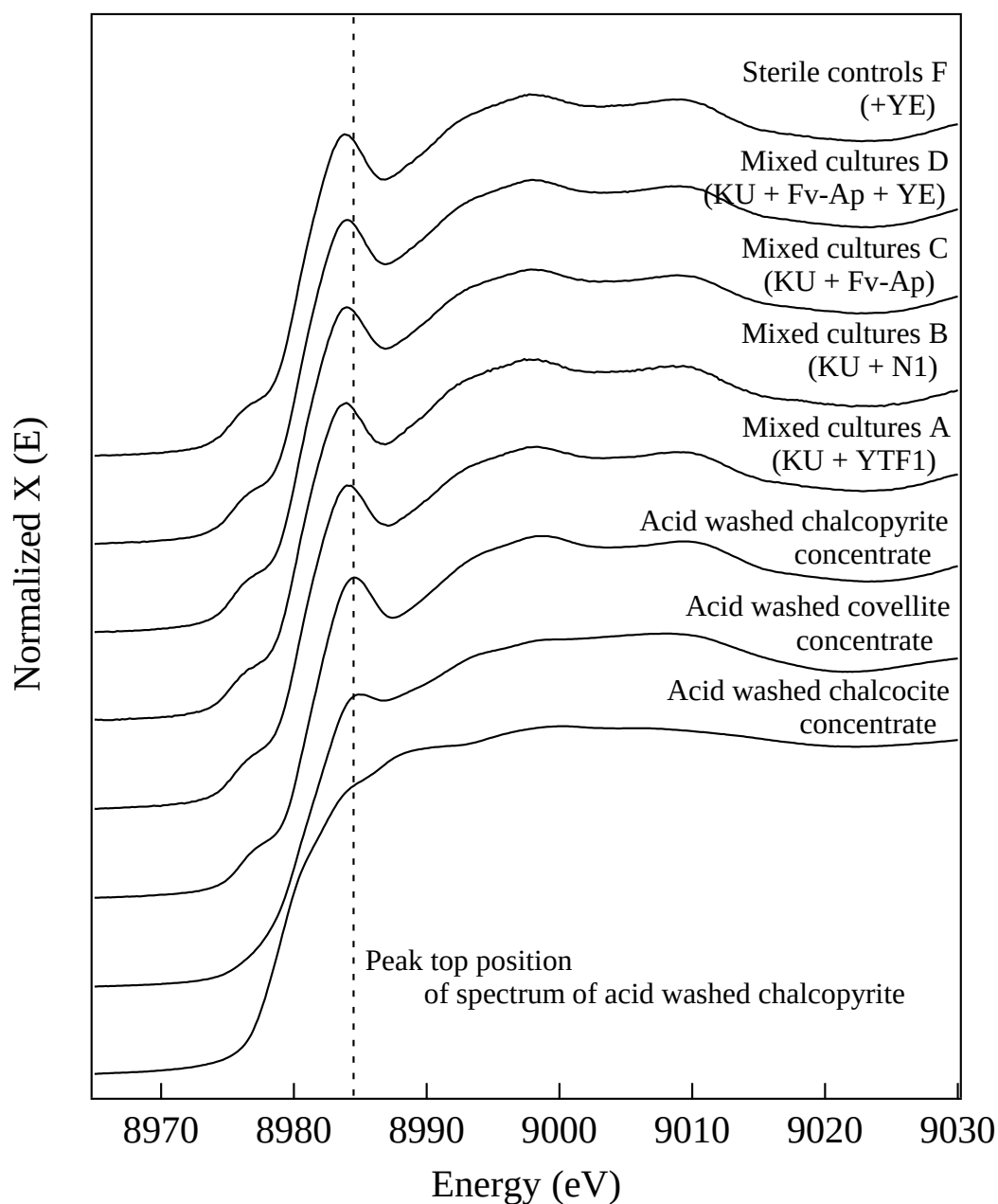


Fig. 8.22 Normalized XANES spectra at the Cu K-edge for acid washed concentrates of chalcocite, covellite, and chalcopyrite and bioleached residues (day 70) of mixed cultures A (KU + YTF1), B (KU + N1), C (KU + Fv-Ap), and D (KU + Fv-Ap, YE), and sterile controls F, from the bottom to the top, with off-set.

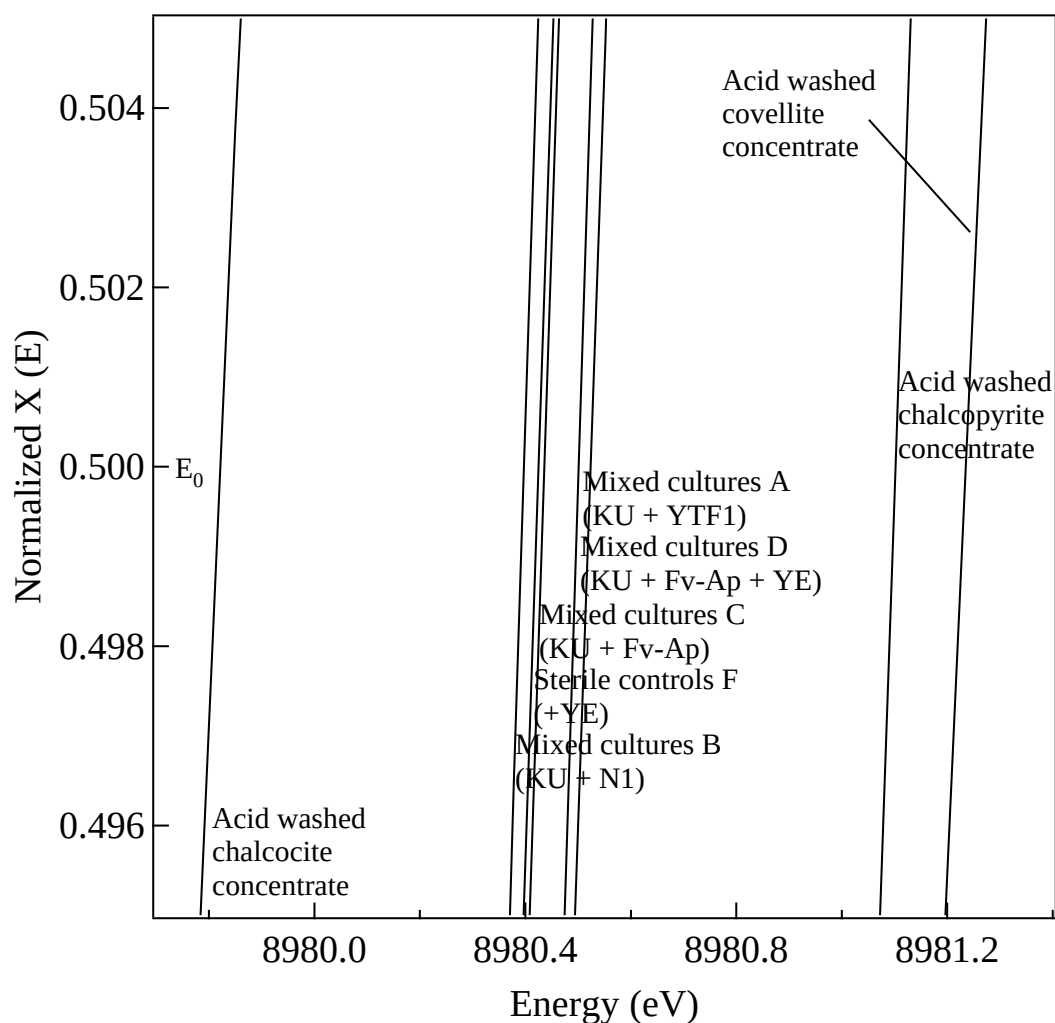


Fig. 8.23 Variation of E_0 values of the XANES spectra at the Cu K-edge for acid washed concentrates of chalcocite, covellite, and chalcopyrite and bioleached residues (day 70) of mixed cultures A (KU + YTF1), B (KU + N1), C (KU + Fv-Ap), and D (KU + Fv-Ap, YE), and sterile controls F.

Table 8.3 Variation of E_0 values of the XANES spectra at the Cu K-edge for acid washed concentrates of chalcocite, covellite, and chalcopyrite and bioleached residues (day 70) of mixed cultures A (KU + YTF1), B (KU + N1), C (KU + Fv-Ap), and D (KU + Fv-Ap, YE), and sterile controls F.

Bioleached residues/ reference samples	Acid washed chalcocite concentrate	Mixed cultures B (KU + N1)	Sterile controls F	Mixed cultures C (KU + Fv-Ap)	Mixed cultures D (KU + Fv-Ap + YE)	Mixed cultures A (KU + YTF1)	Acid washed chalcopyrite concentrate	Acid washed covellite concentrate
E_0 (eV) of Cu K-edge	8979.82	8980.40	8980.42	8980.44	8980.50	8980.52	8981.10	8981.23
Lower → Higher								

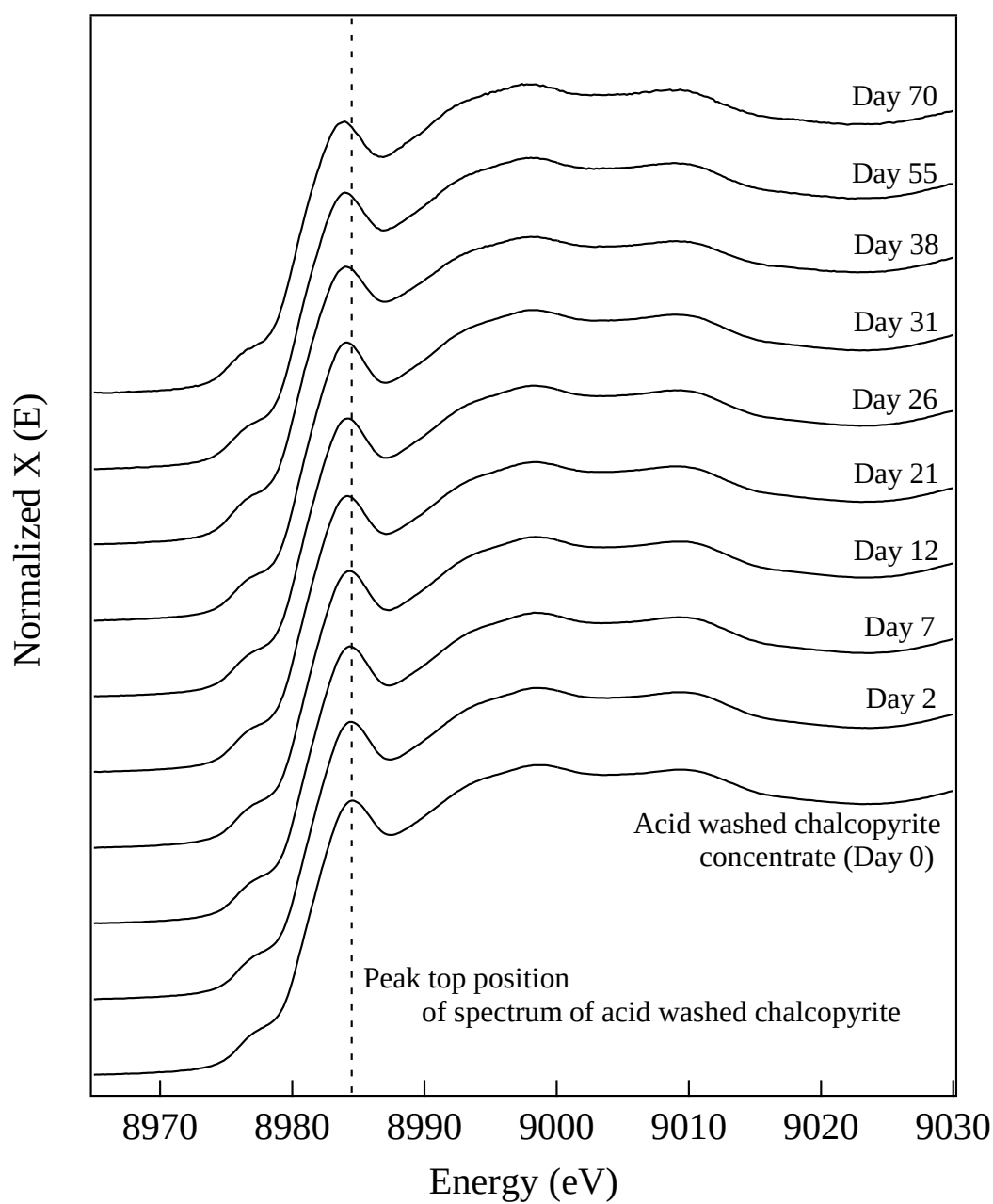


Fig. 8.24 Normalized XANES spectra at the Cu K-edge for acid washed chalcopyrite and bioleached residues collected at day 2, 7, 12, 21, 26, 31, 38, 55, and 70, from the bottom to the top, with off-set.

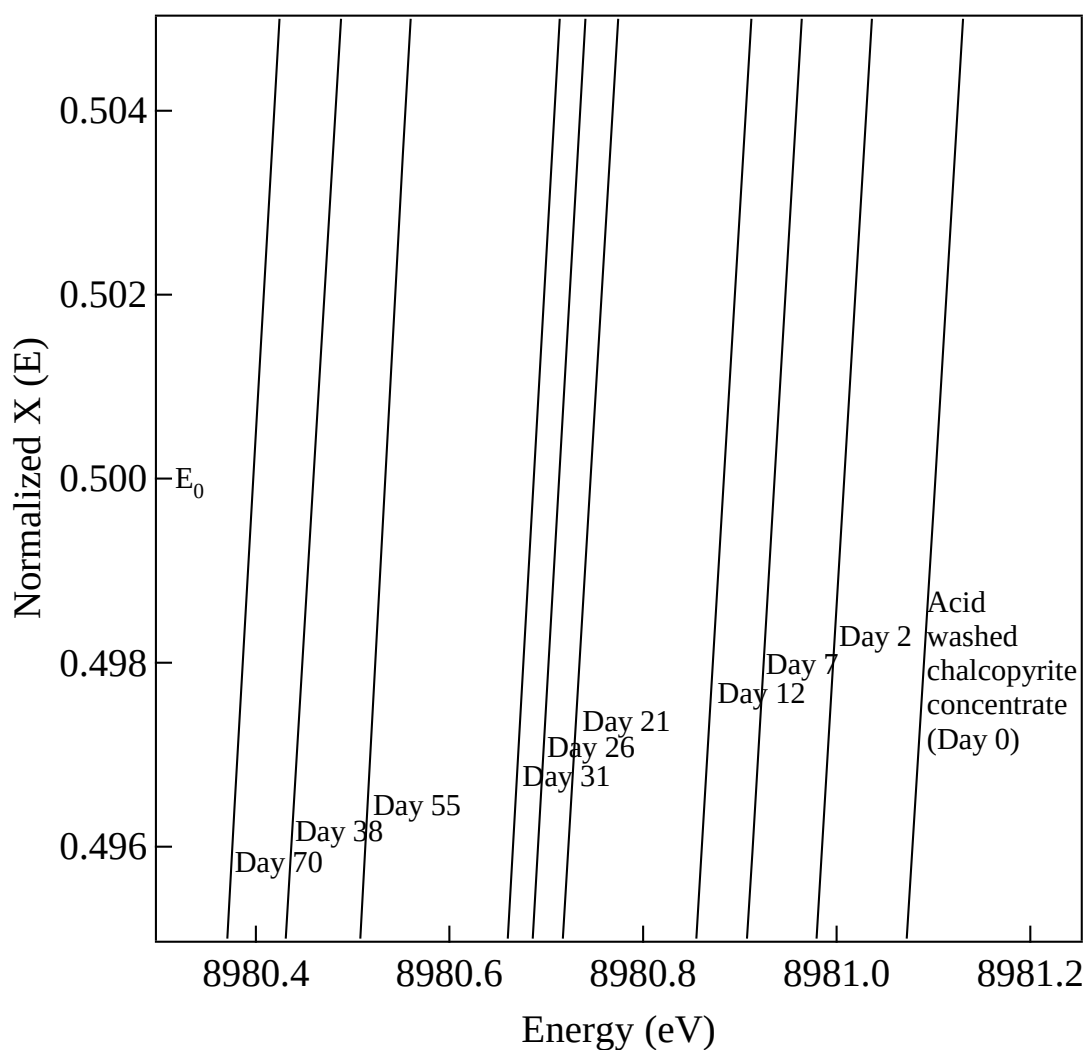


Fig. 8.25 Variation of E_0 values of the XANES spectra at the Cu K-edge for acid washed chalcopyrite (day 0) and bioleached residues collected at day 2, 7, 12, 21, 26, 31, 38, 55, and 70.

Fig. 8.4 Variation of E_0 values of the XANES spectra at the Cu K-edge for acid washed chalcopyrite (day 0) and bioleached residues collected at day 2, 7, 12, 21, 26, 31, 38, 55, and 70.

Bioleached residues/ reference samples	Day 70	Day 38	Day 55	Day 31	Day 26	Day 21	Day 12	Day 7	Day 2	Acid washed chalcopyrite concentrate
E_0 (eV) of Cu K-edge	8980.40	8980.46	8980.53	8980.69	8980.71	8980.75	8980.88	8980.94	8981.01	8981.10
Lower → Higher										

8.4 Conclusions

The effect of microbiological control of solution redox potential on chalcopyrite bioleaching efficiency was investigated using mixed cultures of moderately thermophilic acidophiles (*At. caldus* strain KU mixed with different Fe(II)-oxidizers) in this study. Utilization of different Fe(II)-oxidizing acidophiles to the chalcopyrite bioleaching test resulted in different behaviors of solution redox potentials (the E_{normal} values): Relatively weaker Fe(II)-oxidizing capability of *Sb. acidophilus* strain YTF1 was found to maintain the E_{normal} levels around 0.5 throughout the bioleaching test, while the stronger Fe(II)-oxidizing capability of *Acidiplasma* sp. strain Fv-Ap resulted in excess increase in the E_{normal} level over 1.0. The E_{normal} level was found to critically affect Cu recovery in the chalcopyrite bioleaching system owing to the formation and oxidation of intermediate chalcocite: Higher Cu recovery (75% at day 70) was obtained when the E_{normal} values were maintained within the range $0 < E_{\text{normal}} < 1$, especially in the middle of the range, whereas lower Cu recovery (49% at day 70) was observed when the E_{normal} values were maintained at $E_{\text{normal}} > 1$. When tested with *Sb. sibiricus* strain N1 as an Fe(II)-oxidizer, Cu extraction slowed down after the E_{normal} values increased up to 1.0, indicating that there is no longer involvement of chalcocite at $E_{\text{normal}} > 1$. Furthermore, Cu dissolution within the range $0 < E_{\text{normal}} < 1$ was found to be controlled by surface reactions, whereas that at $E_{\text{normal}} > 1$ was found to be controlled by diffusion through a product-layer. Cu extraction rate via the former model was much efficient rather than that via the latter model.

This study successfully demonstrated the effectiveness of microbiological control of solution redox potential by naturally-occurring microbial Fe(II)-oxidizing ability for maximization of Cu recovery in the chalcopyrite bioleaching system. Optimization of the bioleaching conditions could lead to further improvement in Cu recovery from refractory primary copper sulfide, chalcopyrite.

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Chapter 9

Conclusions and recommendations for future work

9.1 Conclusions

This thesis represents investigations of iron redox abilities of extremely acidophilic microorganisms as fundamental studies, followed by the application studies utilizing microbial iron redox abilities to mineral processing, such as reductive bioleaching of Ni-limonite ore and chalcopyrite, and oxidative bioleaching of chalcopyrite with addition of silver catalyst or microbiological redox potential control.

In chapter 3, with the aim of search for iron-reducing acidophiles possibly applicable to mineral processing applications, the microbial community structure in the Chinoike Jigoku hot spring was first analyzed based on the 16S rRNA gene sequences. Consequently, the presence of Fe metabolizing extreme acidophiles (most closely related to *Acidithiobacillus* sp., *Sulfobacillus* sp. and *Alicyclobacillus* sp.) at the site was found. The isolated strain (identified as *Sulfolobus* sp. with 99% similarity to *S. shibatae* B12, and named *Sulfolobus* sp. GA1) showed unique characteristics; incapable of sulfur oxidation and capable of iron metabolism. The observation motivated us to further investigation of general iron redox abilities in known representative *Sulfolobus* strains, including *S. metallicus* Kra23, *S. tokodaii* 7, *S. acidocaldarius* 98-3, *S. solfataricus* P1, *S. shibatae* B12, and *Sulfolobus* sp. GA1.

In chapter 4, all six representative *Sulfolobus* strains tested were found to oxidize/reduce iron under both aerobic/micro-aerobic and anaerobic conditions. The degree of iron-oxidizing abilities were found to differ largely between the strains. The degree and mechanism of iron oxido-reduction reactions were also found versatile between different species. When hexavalent chromium reduction was tested with the strains (in order to find possible applicability to metal bioremediation), highly-toxic hexavalent chromium was reduced in growth-uncoupled cell-suspensions of all strains. The trend of ferric iron reduction in six strains did not correspond to that of hexavalent chromium, suggesting different mechanisms being involved in ferric iron and hexavalent chromium reduction in *Sulfolobus* spp. Iron redox transformation by *Sulfolobus* spp. may be potentially applicable to bio-hydrometallurgical techniques, including reductive and oxidative bioleaching.

In chapter 5, applicability of the iron-reducing capability in *Sulfolobus* spp., as well as that in several other acidophiles, was investigated by testing with reductive bioleaching of Indonesian nickel-limonite ore. Screening test found the most efficient

iron reduction with *S. tokodaii* 7 among those thermophilic acidophiles tested, and with *Sb. acidophilus* YTF1 among those moderately thermophilic acidophiles tested. Reductive bioleaching of citrate washed-limonite ore using the above two representative strains resulted in improvement in nickel recovery (42-46% at day 30 as a single step), compared with that of sterile controls (26-28% at the same day as a single step). The mechanism here was based on 2-step process: (i) Acid dissolution of the ferric mineral, releasing small amount of soluble Fe(III) in equilibrium with the solid phase mineral, (ii) Rapid reduction of soluble Fe(III) by microorganisms. This “pulls” the acid dissolution phase out of equilibrium, accelerating the rate of mineral dissolution. The amounts of dissolved iron, nickel and aluminum were in liner correlation, but those of magnesium and calcium were not. This indicates that nickel and aluminum were associated with the goethite phase. A negative factor to Ni extraction from the limonite ore was dissolution of calcium contents, indirectly resulting in increase in solution pHs. This was found to negatively affect microbial iron-reducing activity since the resultant pH values exceeded the optimal pH range for the acidophiles used in the experiments. Higher pHs also lead to iron precipitation and thus slowed down goethite dissolution and nickel extraction. Higher recovery of nickel from the limonite ore was achieved by repeating (three times) reductive bioleaching using iron-reducing acidophiles (55% within 30 days). Overall, Ni extraction via Fe dissolution from the Indonesian limonite ore was found to be sensitive to solution redox potential levels, total soluble Fe concentrations, and solution pHs.

The fact that goethite-based limonite ore can be solubilized via reductive bioleaching using microbial iron-reducing capability, motivated us to apply the leaching mechanism to chalcopyrite, refractory primary copper sulfide mineral containing ferric iron (**in chapter 6**). However, no noticeable improvement in copper recovery was observed. In order to apply the reductive bioleaching to ferric containing minerals, shift in chemical equilibrium of iron between solid and solution phases is the crucial key. In the case of chalcopyrite, acid dissolution of ferric iron from the mineral structure hardly occurred due to its higher refractoriness compared with iron oxides such as goethites.

In chapter 7, with respect to improvement in copper recovery from chalcopyrite, refractory primary copper sulfide, effect of silver catalyst (as silver sulfate or silver

sulfide) on its bioleaching efficiency was studied using mixed cultures of iron- and sulfur-oxidizing acidophiles. When tested with the addition of silver sulfate, microbial iron/sulfur-oxidizing capabilities were inhibited by the presence of silver ions and silver sulfide formed. Unexpectedly, after silver sulfate was added at day 10, sterile controls showed higher Cu recovery (52% at day 42) than that in mixed cultures (43% at day 42). This was likely caused by accumulation of elemental sulfur on the chalcopyrite surface formed at higher solution redox potential level in mixed cultures before the addition of silver sulfate. When silver sulfide was added, its inhibitory effect on microbial activity was also confirmed. In silver catalyzed bioleaching of chalcopyrite, continuous microbial re-generation of ferric iron is necessary for silver cycle between silver ions and silver sulfide. However, acidophiles used in the bioleaching experiments was not tolerant to silver ions and silver sulfide. The results obtained in this chapter indicate that a balance between ferrous iron accumulation via silver catalytic effect and microbial iron-oxidizing activity (to maintain E_{normal} levels at the specific range $0 < E_{\text{normal}} < 1$) is important in order to maximize copper recovery in the silver catalyzed-chalcopyrite bioleaching system.

On the basis of redox potential dependence of chalcopyrite dissolution, **in chapter 8**, possible utility of microbiological control of solution redox potential during chalcopyrite bioleaching was investigated using mixed cultures of moderately thermophilic acidophiles (*At. caldus* KU mixed with different iron-oxidizers; *Sb. acidophilus* YTF1, *Sb. sibiricus* N1, and *Acidiplasma* sp. Fv-Ap). Owing to the weakest iron-oxidizing capability of strain YTF1 among those tested, the E_{normal} level was maintained within the specific range between 0 to 1, resulting in the highest copper recovery (75%) by day 70. The lowest recovery was shown in mixed cultures using strain Fv-Ap in the presence of yeast extract (49% at day 70). The copper extraction rate was maximized at the E_{normal} level of approximately 0.3. Chalcopyrite dissolution in the specific range; $0 < E_{\text{normal}} < 1$, was found to be controlled by surface reactions, whereas that at $E_{\text{normal}} > 1$ was found to be controlled by diffusion through a product-layer, with the former more efficient than the latter. Therefore, this study successfully demonstrated the effectiveness of the microbiological solution redox potential control by utilizing naturally-occurring microbial iron-oxidizing ability for improved Cu recovery from chalcopyrite.

Overall, results obtained from fundamental studies in this work (search for iron redox transformation abilities in acidophiles) were applied to the following application studies including reductive bioleaching of limonite ore and oxidative bioleaching with the addition of silver catalyst and microbiological redox potential control. The flow from fundamental to application studies in this work provided us with new aspects and approaches in resources engineering, leading to further development of biological mineral processing technologies.

9.2 Recommendations for future work

As a former part of application studies in this work, dissolution of limonite ores (mainly consisting of goethite) via reductive bioleaching at atmospheric pressure was found to show improvement in nickel recovery. Goethite based-laterite (limonite) is hardly solubilized via organic acid chemical leaching techniques (proposed as alternative approach to dissolve serpentine-based laterite; saprolite). With the advantages being less energy-intensive compared with high pressure acid leaching (HPAL), further studies on the reductive bioleaching of limonite ore would lead to development of low-cost biohydrometallurgical processes. All the experimental data in this work were obtained from batch experiments in laboratory scale and no practical scale tests (e.g., bioreactor tests, column tests, pilot scaled tests) have yet been carried out., In order to establish a general concept of reductive bioleaching as industrial process, a proposed process flowsheet is provided in Fig. 9.1.

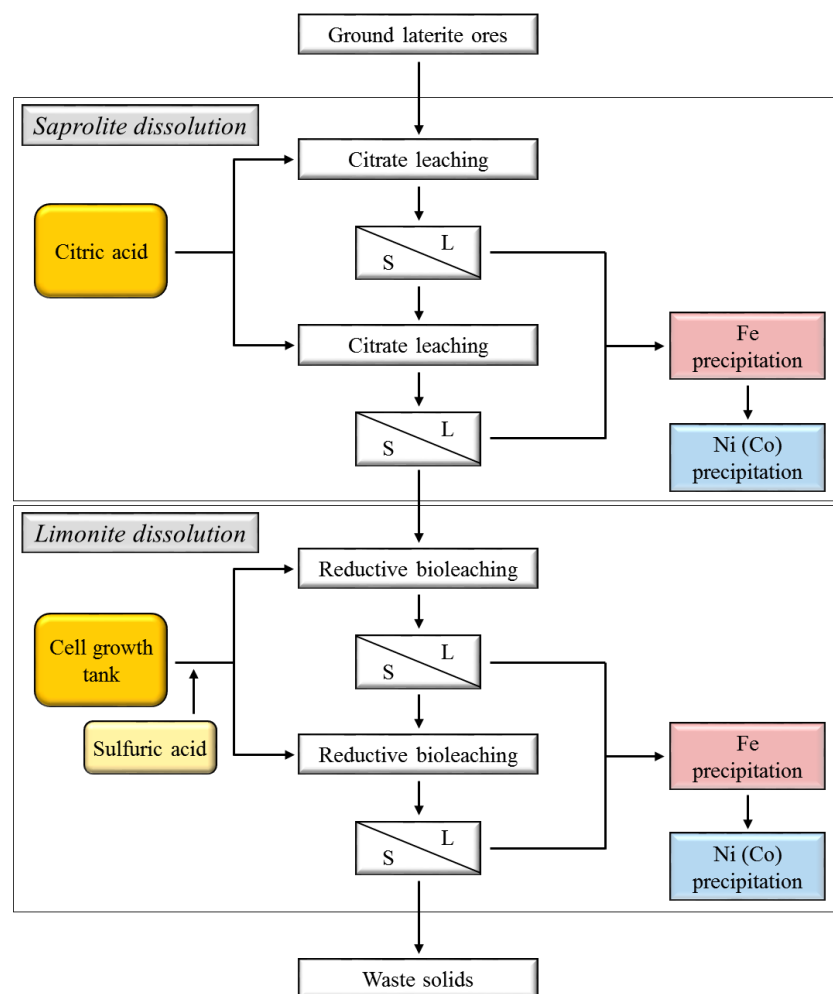


Fig. 9.1 Simplified flowsheet for a proposed nickel recovery process from laterite ore.

In the representative flowsheet of reductive bioleaching of laterite ore, saprolite is first targeted in the former process via citrate leaching. In order to maximize nickel recovery, the chemical leaching is repeated twice. After 1st and 2nd steps of the citrate chemical leaching, the liquid is fed to the iron precipitation treatment, followed by nickel precipitation. Citrate leached-residues are subsequently fed to the sequential reductive bioleaching (1st and 2nd steps): Microorganisms are cultivated in the cell growth tank, and fed to the both reductive bioleaching steps. In the case of reductive bioleaching, the liquid goes to the iron precipitation process, followed by nickel precipitation. At last, bioleached residues are discharged as waste solids containing silicate.

As a latter part of application studies, enhancement of copper recovery in the chalcopyrite bioleaching system was studied. During silver catalyzed-bioleaching test, iron- and sulfur-oxidizing acidophiles used did not show tolerance to silver ions and silver sulfide. Still, positive effect by the addition of silver catalyst on chalcopyrite leaching was observed in sterile controls. Therefore, higher Cu recovery can be expected through silver catalyzed-bioleaching by (i) adapting acidophiles to silver ions in order to maintain high solution redox level and to prevent sulfur accumulation, or (ii) using other acidophiles with higher tolerance to silver ions. Further studies on effect of solution redox levels and silver catalyst on chalcopyrite bioleaching efficiency would lead to development of effective bioleaching technologies for this highly refractory mineral in the near future.

Study on chalcopyrite bioleaching with the microbiological redox potential control took advantage of “weak” iron-oxidation displayed by certain acidophiles. By doing so chalcopyrite dissolution was promoted, leading to efficient copper recovery. One of the advantages of the proposed system is that much less generation of secondary ferric minerals is expected (e.g., jarosite), since the solution redox potentials would be maintained at relatively low level where reaction speed of jarosite formation is relatively low. The general concept of the bioleaching system is proposed in Fig. 9.2.

This is the first report challenging microbiological control of solution redox potential level during the chalcopyrite bioleaching. Therefore, further studies focusing on optimization of the bioleaching conditions (e.g., microbial systems, initial pHs, temperatures, shaking speeds, pulp density, particle sizes, initial concentrations of

ferrous iron/copper ion, using bio-reactors, etc.) will provide higher copper recovery in the system. Since all the experimental data of chalcopyrite bioleaching were obtained from batch experiments in laboratory scale, more practical scaled experiments will be necessary to evaluate applicability of the proposed system.

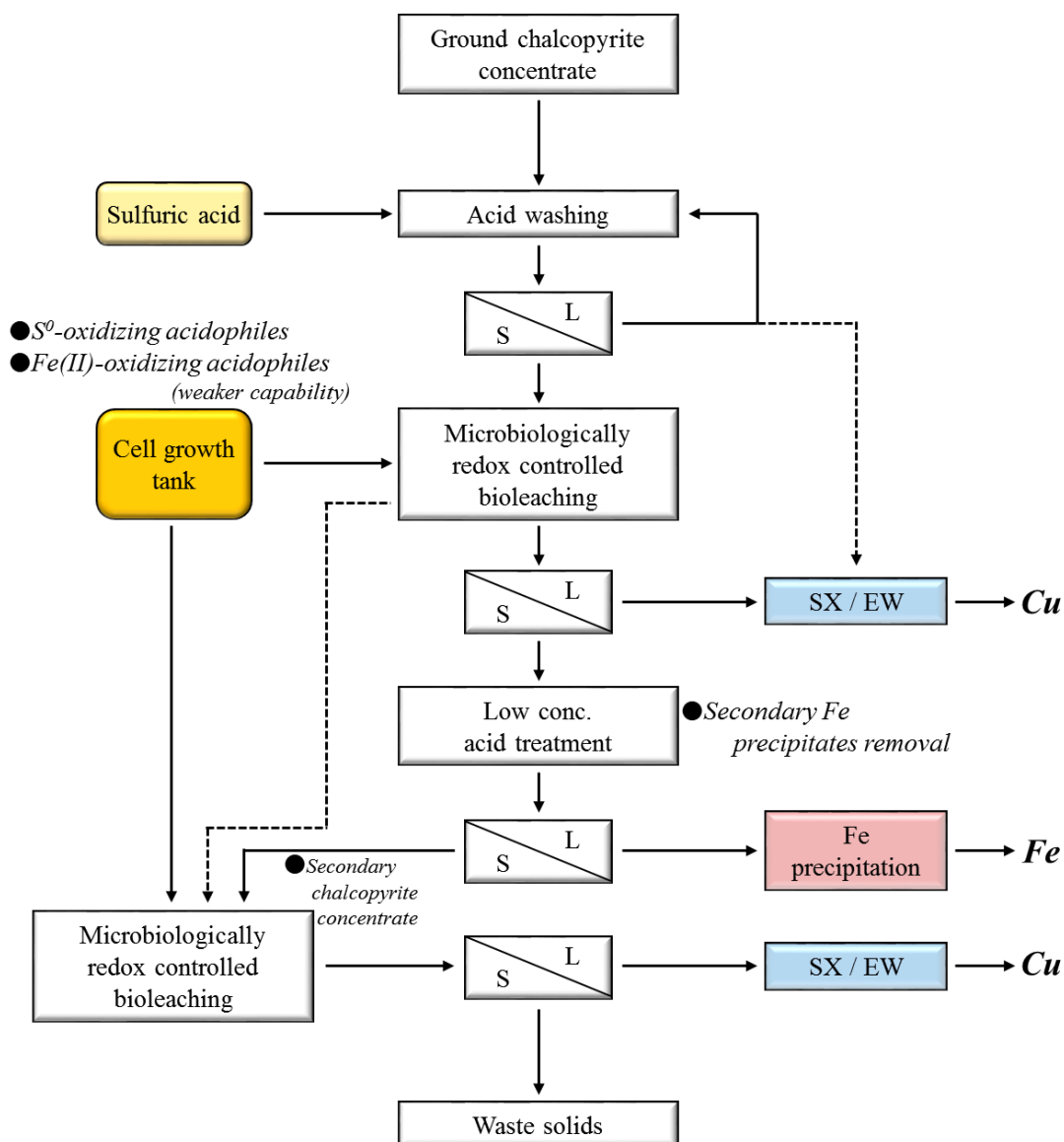


Fig. 9.2 Simplified flowsheet for a proposed copper recovery process from chalcopyrite concentrate.

In the representative flowsheet of chalcopyrite concentrate bioleaching process, , the concentrate is first washed with sulfuric acid to remove acid soluble-impurities after the grinding. The liquid is fed back to the acid washing step, and transferred to SX/EW

unit when soluble copper is accumulated in the acid solution. After that, acid washed-chalcopyrite concentrate is fed to bioleaching system, where the redox potential level is microbiologically maintained at E_{normal} between 0 to 1: Mixed cultures of sulfur- and iron-oxidizing acidophiles are cultivated in the cell growth tank, and fed to the both bioleaching steps. Bioleached residues after the 1st bioleaching step is treated with low concentration sulfuric acid for the purpose of removing ferric iron precipitates produced on the surface of chalcopyrite concentrate, and subsequently transferred to solid/liquid separation. The liquid is fed to the iron precipitation unit, while the liquid goes to the 2nd bioleaching step: bioleaching solution in the 1st bioleaching step is partially fed to the 2nd step in order to make solution conditions in the 2nd step stable. After the solid/liquid separation, leachate containing soluble copper is sent to the SX/EW unit to recovery copper, while residual solid is collected as waste.

A number of reports focusing on bioleaching of sulfide minerals including chalcopyrite from different perspectives have been reported, contributing to further development of industrial bioleaching operation. BHP-Billiton has been developing biological leaching technologies, in particular chalcopyrite bioleaching in a stirred-tank (Olson et al 2003). Bioleaching operation in heaps using thermophilic acidophiles will also become one of the major operational processes in the near future due to a recent increase in production of low grade copper concentrates. It was mentioned by a committee of the United States National Research Council (2002) that the application of biotechnology to the metal extraction is becoming an increasingly important hydrometallurgical processing tool. A number of plants for large-scaled bioleaching operations are currently located especially in developing countries (e.g., Chile, Indonesia, Mexico, Peru and Zambia) given that these countries share over 50% in the world's copper production, and that bioleaching is recognized as a suitable technique for developing countries due to its low capital/operational costs (Gentina et al 1985, Acevedo et al 1993).

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Acknowledgements

It is great pleasure for me to express my gratitude to all people who have supported me to carry out many experiments and to make this thesis. I would like to show feelings of gratitude to committee of my doctoral dissertation examination, including Prof. Keiko Sasaki (as a chief examiner), associate Prof. Naoko Okibe, Prof. Tsuyoshi Hirajima, and Prof. Hiroaki Nakano.

First of all, I would like to show my greatest appreciation to my supervisor associate Prof. Naoko Okibe, for her enormous supports and invaluable suggestions throughout the course of my PhD. I could not finish this work without her help. She has made me what I am. I am very honored to be her first PhD student.

I would like to also thank to Prof. Tsuyoshi Hirajima and Prof. Keiko Sasaki for his important suggestions and constant encouragements. They provided me with critical advices for understanding results obtained from my experiments.

I would like to thank Prof. Hiroaki Nakano from Department of Materials Science and Engineering, Kyushu University as a part of my thesis committee, for his invaluable comments and warm encouragements.

My appreciation goes to also associate Prof. Hajime Miki, associate Prof. Tomoyo Goto, associate Prof. XingHong Qiu, associate Prof. QianQian Yu, and Assistant Prof. Moriyasu Nonaka for his technical advices and insightful comments. In addition, it is needed to thank Mrs. Makiko Semba. This study could not be completed without her technical supports.

I would like to show my appreciation to Prof. David Barrie Johnson from School of Biological Sciences, Bangor University. He kindly accepted my visiting as an international internship student to his laboratory, and provided us with a culture of *Acidiplasma* sp. strain Fv-Ap.

I would like to thank to associate Prof. Maiko Nishibori (Department of Energy and Material Sciences, Kyushu University) for teaching me how to perform XAFS (X-ray absorption fine structure) experiments and to analyze the data, as one of the laboratory rotation program. It is necessary to also thank Mr. Shin-ichi Hirano (CRIEPI; Central Research Institute of Electric Power Industry), Mr. Naoaki Kataoka, and Mr. Takao Hagino (Swing corporation) for accepting me as an internship student

and for taking care of me during the internship. I would like to show my appreciation to associate Prof. Yuichi Sugai (as a tutor) and Ms. Miwa Hirashima and Ms. Minako Matsue (as secretaries) in Advanced Graduate Program in Global Strategy for Green Asia.

I would like to show my acknowledgment to JX Nippon Mining & Metals corporation for providing us with chalcopyrite concentrate.

The XAFS experiments were performed at Kyushu University Beamline (SAGA-LS /BL06) with the proposal of No. 2014IHK025, 2016IK003, and 2016IHK013. The XRF experiments were performed at Center of Advanced Instrumental Analysis, Kyushu University. I would like to thank Mr. Kazuhiko Shimada for his technical supports during the EPMA experiments at Department of Earth and Planetary Sciences, Kyushu University. We appreciate the technical assistance from The Research Support Center, Research Center for Human Disease Modeling, Kyushu University Graduate School of Medical Sciences. Permission for field sampling and photographic images were kindly given by Mr. Masafumi Kudo, manager of the Chinoike Jigoku hot spring.

I would like to express my appreciation to Advanced Graduate Program in Global Strategy for Green Asia. This educational program provided me with meaningful opportunities to learn and understand a relationship between technologies and society. I am also grateful for financial support provided by the program.

I would like to give heartfelt thanks to Lab. Members in Mineral Processing, Recycling and Environmental Remediation Laboratory as follows; Dr. Satoshi Kumagai, Dr. Sayo Moriyama, Dr. Dewi Agustina Iryani, Dr. Limsuwan Pilasinee, Dr. Mohsen M. Farahat, Dr. Ahmed M. Elmahdy, Dr. Paulmanickam Koilraj, Dr. Widi Astuti, Dr. Mutia Dewi Yuniati, Dr. Wuhui Luo, Atsunori Tayaoka, Sho-ichi, Tsuruyama, Yashinobu Nagashima, Keitaro Kitagawa, Naoyuki Fukumoto, Masaharu Koga, Mikoto Koga, Masayuki Kuwata, Emiko Morioka, Masashi Maki, Masanori Mori, Hisaya Tuji, Yukiho Hosomomi, Yuko Misumi, Masahito Tanaka, Shiori Morishita, Taichi Momoki, Osamu Ichikawa, Mari Yoshida, Santisak Kitjanukit, Kojo Twum Konadu, Yuken Fukano, Daisuke Nakayama, Hidekazu Matsuoka, Yu Takaki, Kenta Toshiyuki, Akinobu Iguchi, Katsutoshi Tsutsumi, Takahiro Matsumoto, Keishi Oyama, Tsubasa Oji, Akihiro Inoue, Shugo Nagato, Kazuyoshi Oka, XiangChun Liu, Gde Pandhe Wisnu Suyantara, Binglin Guo, Intan Nurul Rizki, Melisa Pramesti Dewi,

Acknowledgements

Yuta Era, Takeru Fukumori, Taigen Masuyama, Ryota Matsushita, Yusuke Hotta, Kyohei Takamatsu, Yuta Kamura, Yoshikazu Hayashi, Yu hirayama, Yukihiro Muta. It was great pleasure to be together in the laboratory.

Finally, I would also like to express my gratitude to my family for their moral support and warm encouragements.

March 2017, Yusei Masaki